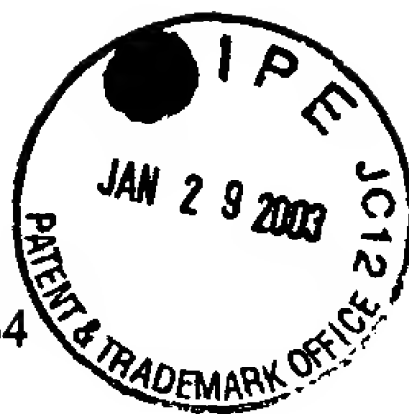


In re Application of:  
Vale et al.

Application No.: 09/502,664

Filed: February 11, 2000

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PATENT  
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## II. REMARKS

Upon entry of the amendment, claims 1, 3 to 12, and 104 to 111 will be pending. A marked-up version showing the amendment to claim 8 is attached as Exhibit A.

Claim 8 is amended herein, and claims 105 to 111 are added. No new matter has been added with the claim amendments and new claims. The amendment to claim 8 replaces trademark names with corresponding chemical names. Newly added claims 105 and 109 are supported by the disclosure at page 13, lines 17 to 19. Newly added claim 106 is supported by the disclosure at page 13, first paragraph. Newly added claim 107 is supported by previously pending claims 1 and 8. Newly added claim 108 is supported by previously pending claim 3. Newly added claim 110 is supported by page 14, lines 7 to 9. Newly added claim 111 is supported by previously pending claim 104.

### Regarding the Declaration

The Office Action objects to the Oath/Declaration because the inventor Mariuia Matuska's citizenship is listed as "German" instead of "Germany." Filed herewith is a newly executed declaration of Mariunia Matuska which recites citizenship as "Germany." Therefore, it is respectfully requested that this objection be withdrawn.

### Rejections under 35 U.S.C. § 112

The Office Action rejects claims 1, 3-12, and 104 under 35 U.S.C. § 112, second paragraph, as allegedly vague and indefinite. Applicants respectfully traverse the rejection.

Claims must particularly point out and distinctly claim the subject matter which the applicants regard as the invention. 35 U.S.C. § 112, second paragraph. The essential inquiry is whether the claims set out and circumscribe a particular subject matter with a reasonable degree of clarity and particularity in light of the specification, the teachings in the prior art, and the claim interpretation of one possessing an ordinary level of skill in the art. MPEP § 2173.02.

The Office Action alleges that claim 1 is indefinite in referring to a "solid support" because the claim does not recite the type of solid support. In response to the Applicants' previous response that the term "solid support" would be clearly understood by one skilled in the art of protein purification to refer to any of various different materials commonly used in the art, the Office Action asserts that the Applicants' contention is precisely why the rejection was made. The Office Action asserts that the claim needs to specify which solid support is pertinent to the claimed invention for one of skill in the art to be able to practice the full scope of the claim.

Applicants respectfully assert that the term "solid support" in claim 1 does not render this claim indefinite. One skilled in the art will recognize that "solid supports" include a variety of solid phase materials known in the art of affinity chromatography (see e.g. page 31, lines 11-27). The availability of a variety of such materials does not render claim 1 indefinite because the skilled artisan would know of many solid supports used in affinity chromatography, any of which can be used as a solid support for linking a material. Therefore, it is maintained that the term does not render the claim indefinite.

The Office Action asserts that because of the many types of known solid supports, a skilled artisan could not practice the full scope of the invention. As stated in MPEP § 2173.04, claim breadth is not indefiniteness. A skilled artisan viewing the specification would recognize that a wide variety of solid supports could be successfully employed with the modified FLAsH compounds of the present invention, and would know that, in addition to absorption and adsorption onto inert solid supports, the reactive amine on the modified FLAsH compounds of the present invention, resulting from acylation with an amino acid, enables the compounds to be readily coupled to a wide variety of solid phases that have been rendered reactive for coupling (page 31, second full paragraph).

A review of issued U.S. patents provides objective evidence that the term "solid support" is known in the art and does not render pending claim 1 indefinite. For example, U.S. Pat. No. 5,415,999 (the '999 Patent)(Exhibit B) which issued on May 16, 1995, claims a method for detecting the presence of an analyte in which a macromolecular ligand is bound to a "solid support" (see dependent claim 2). The specification of the '999 Patent states that:

various substrates can be employed to which the macromolecular ligand will bind, including glass, plastic, or the like. Of particular interest is the use of proteins as the macromolecular ligand, in conjunction with various plastics, e.g. polystyrene, polypropylene, polyethylene, polyolefin copolymers, polycarbonate, methacrylates, PMPP, SAM, and the like, where the protein will strongly adhere to the plastic surface and the solid support is compatible with the assay reagents, conditions and the polymerized polyunsaturated lipid layer.

(U.S. Pat. No. 5,425,999 at column 6, lines 48-58)

Therefore, the '999 Patent provides evidence that the term "solid support" is well known and understood in the art.

As further evidence that the meaning of the term "solid support" is known in the art, U.S. Pat. No. 5,264,557 (Exhibit C), which issued on November 23, 1993, claims a polypeptide bound to a "solid support" but provides no specific examples of solid supports that can be employed. It is submitted that the lack of such examples provides further evidence that the term "solid support" is known in the art. Thus, it is clear from the art that the term "solid support" would be understood to the skilled artisan. As such, it is respectfully requested that this rejection be removed or that, in the alternative, the Examiner provides objective evidence to support the rejection. It is also noteworthy that newly added independent claim 107 recites specific solid supports.

With respect to claim 8, the Office Action alleges that the claim is indefinite because it recites the trademarks Nylon<sup>TM</sup> and Teflon<sup>TM</sup>. Claim 8 is amended herein to recite the chemical names for Nylon<sup>TM</sup> (i.e. polyamide) and Teflon<sup>TM</sup> (i.e. poly(tetrafluoroethylene)). Therefore, the rejection of claim 8 has been overcome. Accordingly, it is respectfully requested that the rejections of claims 1, 3-12, and 14 under 35 U.S.C. § 112, second paragraph, be removed.

### **Prior Art Rejections**

Claims 1, 4 to 7, and 9 to 12 stand rejected under 35 U.S.C. § 102(a) as allegedly anticipated by U.S. Pat. No. 6,008,378 ("the '378 Patent"). Applicants respectfully traverse the rejection. To anticipate an invention, each and every element of a claim must be found in a single prior art reference. MPEP 2131; *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628,631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987).

It is asserted in the Office Action that the '378 Patent teaches a FlAsH compound, and modified forms thereof. The Office Action points to Figures 3, 6, 8, and 9 of the '378 Patent for the teaching of a modified FlAsH compound. In response to Applicants' prior argument that the '378 Patent does not anticipate the present invention because it does not teach or suggest a FlAsH compound which is modified by acylation with an amino acid, the Office Action asserts that the Applicant provided no statement to support how the '378 Patent does not anticipate the present invention.

The '378 Patent, including cited Figures 3, 6, 7, or 9, does not teach or suggest a FlAsH compound which is modified by acylation with an amino acid. This is apparent upon a comparison of the modified FlAsH compound of structure 5 of figure 1 of the subject application, and the structures of figures 3, 6, 7, and 9 of the '378 Patent. In the reaction scheme illustrated in figure 1 of the subject application an acyl group is provided by reaction of the carboxyl group of an amino acid (e.g. beta-alanine) with a primary amine at the 5 position of fluorescein. Examination of moieties that are added to the 5 position of fluorescein on the compounds of the '378 Patent illustrates that the only compound that includes an amino acid at this position is the lower compound of figure 6. However, a careful analysis of this compound and the figure reveals that the amino acid was attached via an  $\epsilon$ -amino group of a lysine residue, not a carboxyl group of the amino acid. Therefore, the acyl group in this compound of the '378 Patent is not provided by acylation with an amino acid, as required of the claimed invention. Accordingly, it is submitted that the '378 Patent does not anticipate the present invention, and, therefore, respectfully requested that the rejection of claims 1, 4 to 7 and 9 to 12 as anticipated by the '378 Patent be removed.

Claims 1, 4 to 7, and 9 to 12 stand rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Griffen et al. (Science, vol. 281, pages 269-272 (1998)). Applicants respectfully traverse the rejection. The Office Action asserts that Griffen et al. teach a FLaSH compound which interacts with proteins tagged with a Cys-Cys-X-X-Cys-Cys sequence, and the use of dithiols such as EDT to facilitate purification of proteins. In response to Applicants' prior argument that Griffen et al. does not anticipate the present invention because it does not teach or suggest a modified FLaSH compound which is modified by acylation with an amino acid, the Office Action asserts that the Applicant provided no statement to support how Griffen et al. does not anticipate the present invention.

Griffen et al. do not anticipate the present invention because, like the '378 Patent, Griffen et al. do not teach or suggest a FLaSH compound that is modified by acylation with an amino acid. The present application acknowledges that Griffen et al. provide a FLaSH compound (See page 3, first paragraph). However, Griffin et al. merely mention FLaSH analogs (page 271, right column top paragraph) but do not teach or suggest any specific FLaSH analogs. In particular, Griffen et al. are silent as to a modified FLaSH compound which is modified by acylation with an amino acid, as claimed. Accordingly, it is submitted that the Griffen et al. reference does not anticipate the present invention. Therefore, Applicants respectfully request that the rejection of claims 1, 4 to 7, and 9 to 12 as anticipated by Griffen et al. be removed.

It is noteworthy that newly added claim 106 recites that the modified FLaSH compound is modified by acylation of a primary amine at a 5 position of fluorescein, using an amino acid. It is submitted that the cited references are silent as to this element. Newly added claims 105, 109, and 110 recite preferred reactive solid supports that are not disclosed in combination with the modified FLaSH compounds of the present invention in any cited reference. Also, newly added claim 107 recites specific solid supports as recited in pending claim 8.

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The Examiner is invited to contact Applicants' undersigned representative if there are any questions relating to this application. Accompanying this response is a petition for two-months extension of time and the required fee. The Commissioner is authorized to charge any additional fees that may be required, or credit any overpayments, to Deposit Account No. 50-1355.

Respectfully submitted,

Dated: January 24, 2003

A handwritten signature in cursive script that reads "Lisa A. Haile".

Lisa A. Haile, J.D., Ph.D.

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Application No.: 09/502,664

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Exhibit A - Page 1



PATENT

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**EXHIBIT A**

**MARKED VERSION SHOWING THE CLAIM AMENDMENTS**

Claim 8 was amended as follows:

8. (Twice amended) The method of claim 1, wherein said solid support is selected from the group consisting of agarose, polyacrylimide, glass, ceramics, natural or synthetic polymeric materials, beads, coverslips, paper, metals, metalloids, polacryloylmorpholide, [various plastics and plastic copolymers such as a Nylon<sup>TM</sup> copolymer, a Teflon<sup>TM</sup> copolymer] polyamide, poly(tetrafluoroethylene), polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polystyrene, polystyrene/latex, polymethacrylate, poly(ethylene terephthalate), rayon, nylon, poly(vinyl butyrate), polyvinylidene difluoride (PVDF), silicones, polyformaldehyde, cellulose, cellulose acetate, nitrocellulose, and controlled-pore glass, aerogels, and affinity exchange resins.

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Exhibit B - Page 1

PATENT

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EXHIBIT B

U.S. Patent Number 5,415,999







US005415999A

**United States Patent** [19]

Saul et al.

[11] Patent Number: **5,415,999**[45] Date of Patent: **May 16, 1995**[54] **FLUORESCENT LIPID  
POLYMER-MACROMOLECULAR LIGAND  
COMPOSITIONS AS DETECTION ELEMENT  
IN LIGAND ASSAYS**

[75] Inventors: Tom Saul, El Granada; Georges Der-Balain; Paul Kenhey, both of Mountain View; Heidi Mathis, Burlingame; Shirley Johnson, Mountain View; Hans Ribi, Hillsborough; Tom witty, Santa Cruz, all of Calif.

[73] Assignee: Biocircuits Corporation, Sunnyvale, Calif.

[21] Appl. No.: 89,975

[22] Filed: Jul. 9, 1993

[51] Int. Cl.<sup>6</sup> ..... G01N 33/53[52] U.S. Cl. .... 435/7.9; 435/7.91;  
435/7.92; 435/7.94; 435/18; 435/21; 435/968;  
436/518; 436/805[58] Field of Search ..... 435/7.9, 7.91, 7.92,  
435/7.94, 18, 21, 968; 436/518, 805[56] **References Cited****U.S. PATENT DOCUMENTS**4,489,133 12/1984 Kornberg ..... 435/7.9  
5,268,305 12/1993 Ribi et al. .... 436/501**OTHER PUBLICATIONS**T. E. Wilson et al., *Langmuir*, vol. 8, No. 10, pp. 2361-2364, 1992.Bader et al., "Polymeric Monolayers and Liposomes as Models for Biomembranes", *Advances in Polymer Science*, 64:1-62 (1985).Hashida et al., "More Useful Maleimide Compounds for the Conjugation of Fab' to Horseradish Peroxidase through Thiol Groups in the Hinge", *J. of Applied Biochemistry*, 6:56-63 (1984).Laguzza et al., "New Antitumor Monoclonal Antibody-Vinca Conjugates LY203725 and Related Compounds: Design, Preparation, and Representative in Vivo Activity", *J. Med. Chem.*, 32:548-555 (1989).Lochner et al., "Photoconduction in Polydiacetylene Multilayer Structures and Single Crystals", *Phys. Stat. Solidi*, 88:653-661 (1978).

Miller et al., "Antibody Properties for Chemically Re-

versible Biosensor Applications", *Anal. Chim. Acta.*, 227:135-143 (1989).O'Shannessey et al., "Specific Conjugation Reactions of the Oligosaccharide Moieties of Immunoglobulins", *J. Appl. Bioch.*, 7:347-355 (1985).Owen et al., "Non-Electrode Biosensors in Clinical Biochemistry", *Ann. Clin. Biochem.*, 22:559-564 (1985).Sugi., "Langmuir-Blodgett Films—a Course Towards Molecular Electronics: a Review", *J. Molecular Electronics*, 1:3-17 (1985).Thompson et al., "Biosensors and Bioprobes", *Trends in Anal. Chem.*, 3(7):173-178 (1984).Aime et al., "Study of the Pressure Effect on Polydiacetylene in Good Solvent," *Synthetic Metals* (1991)41-43 203-207.Batchelder et al., "A Compact Raman Microprobe/Microscope: Analysis of Polydiacetylene Langmuir and Langmuir Blodgett Films," *Makromol. Chem., Macromol. Symp.* (1991) 46: 171-179.Chu & Xu, "Chromatic Transition of Polydiacetylene in Solution," *Acc. Chem. Res.* (1991) 24:384-389.Takeda et al., "Molecular Design for Reversible Phase Transition Systems Based on Polydiacetylenes," *Synthetic Metals* (1991) 41-43: 231.*Primary Examiner*—Toni R. Scheiner*Attorney, Agent, or Firm*—Bertram I. Rowland; Bret E. Field[57] **ABSTRACT**

Methods and compositions are provided for the detection of analytes. The method employs a fluorescence production layer which comprises a fluorescent polymerized polyunsaturated lipid layer in association with a ligand which is a member of a specific binding pair, where the ligand is competitive with the analyte for the complementary binding member or is a complementary binding member. By providing for a fluorescence modulation reagent which binds to the fluorescence production layer in proportion to the amount of analyte in the sample, by measuring the resulting fluorescence after carrying out the assay methodology, the amount of analyte can be determined quantitatively.

6 Claims, No Drawings

# FLUORESCENT LIPID POLYMER-MACROMOLECULAR LIGAND COMPOSITIONS AS DETECTION ELEMENT IN LIGAND ASSAYS

## TECHNICAL FIELD

The field of this invention is the detection of molecules by means of fluorescent materials in association with materials capable of binding to a substance to form a specific binding pair.

## BACKGROUND

As the world has become more complex and as our understanding of different phenomena has increased, there has been a concomitant need to improve methods of measuring the wide variety of substances. From the clinical laboratory, there has been increasing interest in being able to measure various substances in the doctor's office, the home, at bedside, in the field, as well as other sites. With the continuously increasing number of physiologically active substances, both naturally occurring and synthetic, there has been a desire to be able to measure these substances as indicative of the health status of an individual, for therapeutic dosage monitoring, for research, and the like. The substances may be found in a wide variety of samples, ranging over numerous orders of magnitude in concentration for their dynamic ranges of activity, and further differ as to the ease with which one may detect their presence. An area which has only recently assumed substantial commercial importance and will be of increasing importance is the detection of specific nucleotide sequences. Nucleotide sequences find application in genetic counseling, forensic medicine, detection of diseases, and the like. There is, therefore, a wide diversity of opportunities to measure diverse substances from different sources with different sensitivities and for a wide range of purposes.

The methods for detection have ranged from radioactive labels, light absorption, fluorescence, chemiluminescence, agglutination, etc. Each of these methods has found application and has disadvantages as well as advantages over alternative methods. As yet, there has been no single method which has proven applicable in all situations. There is, therefore, substantial interest in devising new methods which may provide for significant opportunities in measuring compounds of interest, where the protocols, apparatus, or reagents may provide advantages over other techniques.

## RELEVANT LITERATURE

U.S. Pat. No. 4,489,133 describes procedures and compositions involving orderly arrays of protein molecules bound to surfactants. Thomas, et al., *Electron. Letters* (1984) 20:83-84 describe a GaAs/LB film MISS switching device employing  $\omega$ -tricosenoic acid as the surfactant bilayer for producing a thin insulator. Lochner, et al., *Phys. Status Solidi* (1978) 88:653-661 describe photoconduction in polydiacetylene multilayer structures and single crystals. Sugi, *J. Molecular Electronics* (1985) 1:3-17 provides a review of Langmuir-Blodgett film use in electronics. Reynolds, *ibid* (1986) 2:1-21 describes conducting organic polymers. Wilson, *Electron. Letters* (1983) 19:237 describes the principles of a three dimensional molecular electronic memory employing polydiacetylene crystals or Langmuir-Blodgett multilayer films. Descriptions of electronic devices employing organized macromolecu-

lar ensembles formed with surfactant layer crystallization include Arrhenius, et al., *Proc. Natl. Acad. Sci. USA* (1986) 83:5355-5359; Haddon and Lamola, *ibid* (1985) 82:1874-1878; and Paleos, *Chem. Soc. Rev.* (1985) 14:45-67. Vandevyer, et al., *J. Chem. Phys.* (1987) 87:6754-6763. U.S. Pat. No. 4,624,761. Fujiki, et al., *Amer. Chem. Society* (1988) 4:320-326. Biegajski, et al., *Amer. Chem. Society* (1988) 4:689-693. Pecherz, et al., *Journal of Molecular Electronics* (1987) 3:129-133. Lando, et al., *Synthetic Metals* (1984) 9:317-327. Day, et al., *Journal of Applied Polymer Science* (1981) 26:1605-1612. Shutt, et al., *Amer. Chem. Society* (1987) 3:460-467. Dhindsa, et al., *Thin Solid Films* (1988) 165:L97-L100. Metzger, et al., *Amer. Chem. Society* (1988) 4:298-304. Fujiki, et al., *Amer. Chem. Society* (1988) 4:320-326. Wohltjen, et al., *IEEE Transactions on Electron Devices* (1985) 32:1170-1174. Wernet, et al., *Semiconducting L-B Films* (1984) 5:157-164. Sugi, et al., *Thin Solid Films* (1987) 152:305-326. Peterson, *Journal of Molecular Electronics* (1986) 2:95-99. Descriptions of methods for immobilizing biological macromolecules on polymerized surfactant films include: O'Shannessey, et al., *J. Appl. Bioch.* (1985) 7:347-355. Hashida, et al., *J. Appl. Biochem.* (1984) 6:56-63. Packard, et al., *Biochem.* (1986) 25:3548-3552. Laguzza, et al., *J. Med. Chem.* (1989) 32:548-555. Jimbo, et al., *Journal of Molecular Electronics* (1988) 4:111-118. Hanifeld, *Science* (1987) 236:450-453. Goundalkar, *Communications* (1984) 36:465-466. Cress, et al., *Amer. Biotech. Lab.* (February 1989) 16-20. Biosensors employing surfactant layer crystallization are described by Owen, *Ann. Clin. Biochem.* (1985) 22:555-564 and Thompson and Krull, *Trends in Anal. Chem.* (1984) 3(7):173-178. Bader, et al., *Advances in Polymer Sci.* (1985) 64:1-62 describe polymeric monolayers in liposomes as models for biomembranes.

Miller and Anderson, *Anal. Chim. Acta.* (1989) 227:135-143, described a fiber-optic sensor based on a homogeneous fluorescence energy-transfer immunoassay and the chemical kinetics.

## SUMMARY OF THE INVENTION

Sensors are provided comprising a fluorescent layer in conjunction with non-covalently bound specific binding pair members, where the fluorescent layer of particular interest is a conjugated polyunsaturated lipid extended chain. The fluorescent layer is employed in association with macromolecular ligands which are conveniently non-covalently associated with the fluorescent layer. Assays are performed, where a reagent is employed which modulates the fluorescent properties of the fluorescent layer. By employing either competitive or sandwich assays, the modulation of the fluorescence may be related to the amount of analyte in a sample. By employing a fluorimeter, the fluorescence may be measured and related to the amount of analyte.

## DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods and compositions are provided employing a sensor for the detection of analytes. Central to the methods and compositions is a fluorescent layer, particularly a monolayer comprising polymerized lipids, polymerized through double and triple bonds to form an extended polyunsaturated chain which is fluorescent and whose fluorescence may be modulated by appropriate reagents,

The polymerized polyunsaturated lipid layer can serve as a transducer for amplifying a signal resulting from an assay component which affects the fluorescence of the layer. Thus, an agent which may serve as a chromophore, light absorber or scatterer, fluorophore or which agent has other properties which may affect the fluorescence can be measured more sensitively, by the amplification resulting from the interaction of such agent and the polymerized polyunsaturated lipid layer.

The capability of the polymerized polyunsaturated lipid layer may be exemplified by using the polymerized polyunsaturated lipid layer on a support and bringing the polymerized polyunsaturated lipid layer in proximity to a fluorescence modulation reagent capable of influencing the fluorescence of the lipid layer, either directly or indirectly, and measuring the fluorescence of the lipid layer as compared to a known amount of fluorescence modulation reagent in proximity to the polymerized polyunsaturated lipid layer, including the substantial absence of the fluorescence modulation reagent. The fluorescence modulation reagent will be non-covalently associated with the polymerized polyunsaturated lipid layer in sufficient proximity so as to affect the response of the polymerized polyunsaturated lipid layer to light irradiation. By relating the amount of the fluorescence modulation reagent in proximity to the polymerized polyunsaturated lipid layer to an analyte of interest, the fluorescence observed can be quantitatively related to the amount of analyte in a sample. The polymerized lipid layer can be physically separated from the analyte being assayed such that the binding event leads to the production of an effector which modulates the lipid layer's fluorescence.

For assays, in addition to the polymerized lipid layer are macromolecular ligands which can compete with an analyte for binding to a reagent (competitive mode) or bind to the analyte so as to bind the analyte in association with the fluorescent layer (sandwich mode), where the analyte may then act as a bridge to bind the moiety. (The combination of polymerized polyunsaturated lipid layer and macromolecular ligand will be referred to as the "fluorescence production layer.") The macromolecular ligand has at least one binding site of interest. The fluorescence modulation reagent may be one or more molecules and includes a specific moiety that can interact with the polymerized polyunsaturated lipid layer when in proximity to the polymerized polyunsaturated lipid layer and which serves to modulate the fluorescence of the polymerized polyunsaturated lipid layer, usually resulting in a reduction in fluorescence in proportion to the amount of analyte in a sample.

In carrying out the assays, a sample, which may have been subjected to pre-treatment, is added to the fluorescence production layer and incubated for sufficient time for binding to occur, where the fluorescence modulating reagent may be present or absent. Where the assay is a competition assay in that the ligand present in the fluorescence production layer competes with analyte in the sample for the fluorescence modulation reagent, the sample may be combined concomitantly with the fluorescence modulation reagent and the fluorescence production layer or have been combined with the fluorescence modulation reagent prior to addition to the fluorescence production layer. Where the assay is a sandwich assay, in that the analyte serves as a bridge between the fluorescence production layer and the fluorescence modulation reagent, then the sample and fluorescence modulation reagent may be added concur-

rently or consecutively in any order. After incubation for sufficient time for binding to occur, as appropriate, additional reagents may be added, followed by a determination of the fluorescence of the fluorescence production layer.

For the formation of lipid films, a temperature gradient technique may be employed (see U.S. application Ser. Nos. 366,651, filed 06/15/89 and 453,784, filed 12/20/89). Surfactant, including amphiphiles, films may be formed on the surface of an aqueous subphase by standard lipid monolayer technologies. A solution containing a monomeric surfactant composition, dissolved in an organic solvent, is applied to the subphase surface by a micropipet. Solvents may include hydrocarbons such as pentane, hexane, heptane, and decane or chlorocarbons such as mono-, di-, tri- or tetrachloroethane. The subphase may be composed of pure water, glycerol, polyethylene glycol, or other polar organic solvents miscible with water.

Monomeric polymerizable surfactants are spread on the subphase at a concentration ranging from 0.01 to 50 milligrams/milliliter of spreading solvent. The aqueous medium may be pre-heated to disperse the surfactant usually to a temperature of not more than about 130° C., which results in evaporation of the solvent. The medium is then allowed to cool to below room temperature, usually to below about 20° C. The rate of cooling may be regulated by the traverse rate of the subphase slide from the heating element to the cooling element, where typical traverse rates vary from 1 cm/second to 1 cm/minute. When the subphase temperature is raised above the surfactant melting temperature, the non-polymerized regions of film will become fluid. When the subphase is cooled back below the surfactant melting transition, crystals of monomers nucleate from the crystalline polymer region.

The surfactant is then polymerized employing any convenient initiation system, e.g., ultra-violet light. The rate of polymerization will be related to the light intensity, so that shorter times will be employed with higher intensity irradiation. Polymerization times between 0.1 seconds to 3 minutes are generally satisfactory.

The film quality can be inspected optically using methods such as polarization birefringence, lateral diffusion techniques including lateral film pressure, or fluorescence measurements such as fluorescence recovery after photobleaching. Films are inspected for defects, crystal domain size and shape, and integrity. The film may be transferred to different substrates for production of the sensor. Transfer is typically accomplished by slowly lowering a transfer substrate planar to the surfactant film at the air/subphase interface. Contact is made at one end of the subphase surface and then full transfer is accomplished by decreasing the contact angle to zero. Transfer may also be achieved by applying standard Langmuir-Blodgett methods [George L. Gaines Jr.: Insoluble Monolayers at Liquid Gas Interfaces, Interscience Publishers, I. Prigogine Editor, John Wiley and Sons (1964)].

The polymerizable surfactants have been extensively described in the literature as evidenced by the prior art described previously. The composition of the surfactant layer may be homogeneous where the surfactant is polymerizable and has a polar terminus, or heterogeneous, where a mixture of surfactants are employed, some of which are polymerizable and others which are not polymerizable.

The surfactant molecule may have a single lipid chain, e.g., a diynoic acid or a plurality of lipid chains, e.g., diester glycerides, preferably a single chain, and generally not more than two lipid chains. Of particular interest are diynoic amides of dihydroxyamine, particularly of from about 20 to 30 carbon atoms, more particularly of from about 22 to 26 carbon atoms.

Illustrative surfactants include N-(1,2-dihydroxy-3-propyl) pentacosyl-10,12-diynamide, ethanolamino-10,12-pentacosadiynamide, 6,8-hexadecadiynoic acid, 2-hydroxyethyl octadeca-8-10-diynoate, eicosa-12,14-diynyl-10,12-phosphatidyl serine, pentaecosa-10,12-diynoic acid, tricosyl-10,12-diynoic acid, acetylene compounds with multiple diyne groups and other polymer surfactants including single acyl chain polymerizable surfactants.

Various other surfactants may be present as diluents for the polymerizable surfactant. These surfactants may be naturally occurring, synthetic, or combinations thereof, and may be illustrated by laurate, stearate, arachidonate, cholesterol, bile acids, gangliosides, sphingomyelins, cerebroside, or the like.

Various functional groups may be present in the film to provide for polymerization, which allow for Förster energy transfer. For the most part, the functional groups will comprise diynes, although other unsaturated molecules may find use, such as activated diynes, e.g.,  $\alpha$ -ketodienes.

For the most part, the hydrophobic portion of the surfactant will have a chain of at least 6 aliphatic carbon atoms, usually a straight chain of at least 6 aliphatic carbon atoms, and generally not more than a total of about 100 carbon atoms, usually not more than about 34 carbon atoms. Preferably, the number of carbon atoms in the hydrophobic portion of the chain will vary from about 12 to 32, more usually 20 to 30, and more preferably 23 to 28 carbon atoms.

The lipid molecules will terminate in a hydrophilic moiety, cationic, anionic or neutral (nonionic) where the functionalities may include non-oxo carbonyl, e.g., carboxylic acids, esters and amides, oxo-carbonyl, such as diols, aldehydes or ketones, oxy, such as ethers, polyethers, and hydroxyl, amino, such as primary, secondary, and tertiary amines and ammonium, phosphorus acids esters and amide, such as phosphate, phosphonate, and phosphonamide, sulfur functionalities, such as thiol, sulfonates, sulfate, and sulfonamides, and the like. Usually, the polymerizable functionality will be separated from the polar and non-polar termini by at least one carbon atom, generally from about 1 to 50 carbon atoms, more usually from about 1 to 8 carbon atoms. The polymerizable group is typically incorporated into the hydrophobic interior of the surfactant film. Diacetylenic groups are typically incorporated in the hydrocarbon chain of the surfactant so that more than one group is present for polymerization. By having two or more polymerizable groups in the surfactant chain, a multiplicity of electrically conducting and/or optically active polymers may be obtained. This configuration leads to films of higher structural integrity and mechanical strength.

Variations of the headgroup provide for improved film quality, such as stability of the film, surface charge, control of inter-head-group hydrogen bonding, reduction of non-specific binding or fluid matrix effects, and ease of chemical modifications. The hydrocarbon tail of the surfactant may also terminate in a hydrophilic group so that the surfactant is bipolar. [Sher, *Justus Liebigs*

*Ann. Chem.* (1954) 589:234; and Akimoto, et al. *Angew. Chem.* (1981) 20(1):91].

The macromolecular ligand which is non-covalently associated with the lipid layer to form the fluorescent production layer, can be any convenient molecule, which is greater than 5 kD, usually at least about 10 kD molecular weight, more usually at least about 15 kD, and will generally be not more than about 1000 kD, more usually not more than about 500 kD. The ligand is characterized by being a member of a specific binding pair which is able to compete with the analyte for the complementary member of the specific binding pair or is able to bind to the analyte, where the analyte is the complementary binding member. Where the analyte is a macromolecule, the analyte may serve as the ligand for association with the lipid layer. Where the analyte molecular weight is below about 10 kD, particularly below about 5 kD, e.g. a hapten, the haptenic analyte or molecule having competitive binding characteristics, will be bound, usually covalently bound, to a macromolecule of at least about 10 kD, frequently, 15 kD or more. The number of haptenic molecules bound will be at least 1, usually at least 2, generally not more than about 1 per 5 kD, more usually not more than about 1 per 10 kD. Manners of conjugating a wide variety of analytes of interest are extensively described in the literature, the particular manner in which ligands are bound to the macromolecular molecule is not critical to this invention.

For the most part, the macromolecular ligand will be a polymer, conveniently a protein, although other polymeric molecules may be employed, both naturally-occurring or synthetic. Various polymeric compositions include nucleic acids, polysaccharides, hyaluronic acids, polysiloxanes, polyacrylates, etc. The macromolecular ligand will either bind to the lipid layer, the solid support supporting the lipid layer, or both, so as to provide a stable association during the course of the use of the lipid layer.

The manner in which the fluorescence production layer is formed can be varied widely, where the two components can be combined by any convenient means, which provides for retention of the macromolecular ligand in association with the lipid layer. For example, the macromolecular ligand may be coated onto a solid support, where it becomes bound, either covalently or non-covalently, and the lipid layer deposited over the macromolecular layer. Various substrates can be employed to which the macromolecular ligand will bind, including glass, plastic, or the like. Of particular interest is the use of proteins as the macromolecular ligand, in conjunction with various plastics, e.g. polystyrene, polypropylene, polyethylene, polyolefin copolymers, polycarbonate, methacrylates, PMPP, SAM, and the like, where the protein will strongly adhere to the plastic surface and the solid support is compatible with the assay reagents, conditions and the polymerized polyunsaturated lipid layer. Instead of having the macromolecular ligand bound to the substrate initially, the lipid layer may first be applied and adhered to the substrate, followed by addition of the macromolecular ligand in an appropriate liquid medium to the lipid layer. In either event, the macromolecular ligand becomes bound to the substrate and/or fluorescent layer, usually the substrate, since the lipid layer will allow the macromolecular ligand to contact the substrate.

In many instances, the macromolecular ligand may be a receptor molecule, which binds to the analyte. A wide



variety of receptor molecules are available, which primarily include antibodies or binding fragments thereof, e.g. Fab, F(ab')<sub>2</sub>, or the like, enzymes, lectins, and for the purposes of this invention, nucleic acids. Thus, these receptors may serve to bind the analyte, which will be the complementary member of the specific binding pair.

The amount of macromolecular ligand will generally be coated on about 0.01 to 100%, more usually 1 to 10% of the solid surface. So long as there is a sufficient amount of the macromolecular ligand to ensure that the amount of macromolecular ligand will not be limiting in measuring the amount of analyte.

The macromolecular ligand may be applied to the substrate in an appropriate buffered medium, generally at pH in the range from about 2-10, more usually about 5-9. The concentration of macromolecular ligand in the medium will generally be at least about 0.01 µg/ml and not more than about 100 µg/ml, more usually being from about 0.10-5 µg/ml, where the amount of macromolecular ligand may be substantially in excess of the amount that can be absorbed by the substrate. The amount of macromolecular ligand which is bound to the substrate will vary depending upon the manner of application, the nature of the macromolecular ligand and analyte, the dynamic range of the analyte, the fluorescent modulating reagent, and the like.

The manner of coating may be dipping, spraying, brushing, rolling, or the like. The surface to be coated, preferably uncontaminated, will be completely exposed to the medium and incubated for sufficient time, generally less than about 1 min to ensure that there is substantially complete coating of at least a portion of the substrate surface. Since the opposite surface will not be exposed to the assay medium, where the solid support is a slide, the slide may be submerged in the medium. Generally, the temperature will be in the range of about 1°-50° C., more usually about 10°-40° C. After the surface has been exposed for sufficient time for binding to occur, the surface may be removed from the macromolecular ligand containing medium, washed with water, particularly distilled water or deionized water, and then allowed to dry or used directly for the lipid film transfer. Where the lipid film transfer occurs prior to the macromolecular ligand binding, the solid support with the lipid film may be treated in substantially the same way as described above for binding of the macromolecular ligand.

The fluorescent lipid films can be formed at the gas-liquid interface and then transferred to the solid support. Transfer can be readily achieved by using conventional Langmuir-Blodgett conditions. The thickness of the solid support supporting the lipid layer will generally be from about 5-100 mil. The solid support provides the desired structural support. Depending upon the nature of the solid support, it may be desirable to modify the solid support surface to provide for the binding of the macromolecular ligand and lipid film to the surface. Desirably, for glass, the surface may be silanized in accordance with conventional ways. For plastic, chemically reactive groups may be used to functionalize the surface, e.g. chloroalkyl, iminoalkyl, acyl, amino, formyl, etc.

Once the fluorescence production layer is formed on the solid support, it may then be used for detection of an analyte.

In assays, the fluorescence modulation reagent will include a binding member of the specific binding pair, which member either competes with the analyte for

binding to the macromolecular ligand or binds to the analyte bound to the macromolecular ligand. The specific binding member may be conjugated to a quencher chromophore, where the absorption of the quencher overlaps the emission band of the fluorescence of the polymerized polyunsaturated lipid layer or, preferably, to an enzyme which produces a product which can affect the fluorescence of the lipid layer, particularly quenching the fluorescence.

Quenching can be achieved in a number of ways. The most straight-forward way is to have a product which is a dye which absorbs the fluorescence emission light. Thus, where the dye absorbs the emission light, particularly where it is deposited on the lipid layer, there will be a substantial reduction in fluorescence. Alternatively, the product may be a fluorophore which has an absorption band which overlaps the emission band of the polymerized polyunsaturated lipid layer. In this manner, by reading fluorescence in the emission band of the lipid layer, particularly at or about the wavelength range of the peak emission, a reduction in emission intensity, as compared to the absence of the product will provide a measure of the amount of analyte. Alternatively, one may read the fluorescence of the product by measuring the emitted light at or about the emission maximum of the product, where the intensity of emission will be related to the amount of analyte in the sample.

A wide variety of enzymes may be employed in the conjugate. Of particular interest are hydrolases, which allow for production of a fluorescent product or an absorptive dye from a leuco dye. There are many chromophores which may be functionalized as ethers or esters, where the chromophoric properties are substantially different from the unfunctionalized compound. Of particular interest are phenolic compounds which have strong absorptive properties as the free phenol, but are weakly or non-absorptive in the absorption band of the free phenol when functionalized. Thus, one can prepare galactosidyl ethers and use β-galactosidase, phosphate esters and use alkaline phosphatase, sulfate esters and use sulfatases, sialic acid derivatives and use neuraminidase, etc. Various quenchers of interest include fluorescein, umbelliferone, phycobilliprotein, etc.

The manner of conjugation is not critical to this invention, there being numerous illustrations in the literature of conjugation of compounds to enzymes. Where the analyte is a protein, by employing recombinant technology, one may provide for fused proteins comprising the analyte and the enzyme or enzyme subunit. Otherwise, various known linking groups may be employed which allow for covalent binding of the analyte to the enzyme, e.g. glutaraldehyde, maleimidobenzene-sulfonate or carboxylate ester, Ellman's reagent, etc. For fluorescent molecules, various known linking groups may be employed for linking the fluorophore to the analyte. Thus, depending upon the nature of the analyte, the particular linkage will vary widely. In conjugating the analyte to the fluorophore or enzyme, it is important that the conjugate be able to bind to the analyte and, as appropriate, the macromolecular ligand.

In carrying out the assay, the sample, used directly or subject to pre-treatment, may be applied to the fluorescence production layer. Various pre-treatments may be involved, such as removal of cells, extraction, dilution, heating, addition of a variety of releasing reagents, or the like. For the most part, the samples will be physiological samples, such as blood, urine, cerebro-spinal

fluid, saliva, milk, and the like. However, in many other situations, the samples may be derived from process stream effluents, water, air, soil, or other environmental material, animal tissue, human tissue, and the like. In some instances, the analyte of interest may involve particles, such as virus particles, cells, organelles, microsomes, and the like. The significant factor is that the analyte can be dispersed substantially homogeneously and be maintained dispersed in the assay medium during the period of the assay or may be spread over the surface of the fluorescence production layer and non-specific components removed by washing.

The fluorescence modulation reagent may be combined with the sample and other components of the assay medium, e.g. buffer, prior to contact with the fluorescence reduction layer or subsequent to the contact of the assay medium with the fluorescence production layer. In some instances, the fluorescence modulation reagent may be combined with the fluorescence production layer prior to the addition of sample. Of particular interest is where the fluorescence production layer is combined with the sample under conditions, usually anhydrous conditions, where the fluorescence modulation reagent will not react with the fluorescence production layer. By addition of sample, the fluorescence modulation reagent becomes dissolved and may then react in the assay medium as appropriate. In this manner, measurement of the amount of fluorescence modulation reagent to be added may be avoided.

The amount of fluorescence modulation reagent will vary, depending upon whether the assay is a competitive assay or sandwich assay. In the case of the competitive assay, the amount of reagent will vary depending upon the concentration range of interest of the analyte. It may vary from about 0.1, more usually 0.5 times the lowest concentration in the range of interest of analyte to not more than about 10 times, usually not more than about 5 times the highest concentration in the range of interest of the analyte. The particular amount chosen will depend upon a number of factors, such as the rate of binding of the reagent to analyte, the protocol employed, e.g. time of measurement, the range of interest, the sensitivity desired, and the like. In most instances, the particular concentration will be optimized as to a particular analyte.

After addition of the sample in the assay medium to the fluorescence production layer, in the absence of the fluorescence modulation reagent, the medium may be incubated for sufficient time to ensure complete binding. One may then wash to remove any non-specific binding materials present in the assay medium. Conveniently, the same or different buffer solution used for the assay medium may be employed for the washings. Usually, the washings will involve volumes not greater than 10 times, usually not greater than about 5 times the volume of the original assay medium. In the competitive mode, the washings may be followed by the addition of the fluorescence modulation reagent solution, conveniently in an appropriately buffered medium, and the system incubated for sufficient time for the fluorescence modulation reagent to bind to analyte bound to the fluorescence production layer. Non-specifically bound reagent may then be removed by washing. For the enzyme, this will be followed by the addition of substrate where a timed reaction will be performed. One can have a single-point determination, where the determination is made at a fixed time from the addition of the substrate to the enzyme conjugate, or can choose

a rate of reaction, where the variation in fluorescence over a pre-determined timed interval may be used to determine the amount of conjugate bound to the fluorescence production layer.

Any analyte can be determined by the subject method. Ligands, such as haptens and antigens may be determined, where the ligands may include naturally-occurring or synthetic organic molecules, proteins, saccharides, nucleic acids, lipids, or combinations thereof. The ligands may be haptenic or antigenic, single molecules, polysubunit molecules, or aggregations, such as microsomes, cells, virus particles, or the like. The ligands may include various drugs, such as drugs of abuse, therapeutic drugs, toxins, or the like. The analytes may include surface membrane proteins, such as cluster designation proteins, HLA proteins, mutant proteins, lipopolysaccharides, peptide drugs, cancer markers, viral proteins, cyclodextrins, placental antigens, such as TSH, PTH, CEA, AFP, PJA, and PSA, ferritin, interferon, enzymes, cytoplasmic proteins, e.g. transcription factors, elongation factors, ribosomes, etc. Other ligands of interest may include hormones, such as thyroxine, triiodothyronine, growth hormone, steroids, vitamins, cofactors, etc.

Kits can be provided for use in the subject methodology, where the kits would comprise the polymerized polyunsaturated lipid layer on a solid support and the macromolecular ligand, conveniently in proximity to the layer and bound to the support. Also included would be the fluorescence modulation reagent, which may be the analyte or the complementary binding member bound to a fluorescent molecule or bound to an enzyme.

The following examples are offered by way illustration and not by way limitation.

## EXPERIMENTAL

### Example 1

#### Preparation of Thyroxine -Bovine Serum Albumin Conjugate (BSA-T<sub>4</sub>)

A. L-thyroxine (5.0 g) was dispersed in 200 ml methanol, the solution saturated with gaseous HCl, allowed to stand overnight, at which time a precipitate formed. The supernatant was diluted with 400ml diethyl ether, chilled and the precipitate isolated. The precipitate was then suspended in 10:1 methylene chloride/methanol, enough triethylamine added to make the solution clear on sonication, followed by passing the solution through a 50 mm×200 mm silica gel column in 20:1 methylene/methanol and fractions containing the product isolated and stripped of solvent.

B. The T<sub>4</sub> methyl ester prepared above (8.3 g) was combined with 1.16 g triethylamine and 200 ml of chloroform and sonicated to dissolve the ester, followed by cooling in an ice bath. To the ester solution was added a solution of 1.28 g of diglycolic acid anhydrous in 300 ml of chloroform over about 1 h. After the solution was warmed to room temperature, it was stirred overnight. The reaction was monitored with TLC and upon completion, the solvent was stripped and the product passed through a 70 mm×200 mm silica gel column in 50:10:1 methylene chloride/methanol/acetic acid. Fractions containing the product were isolated, the solvent stripped and the precipitate washed with acetone.

C. Into 30 ml of DMF was added the T<sub>4</sub> diglycolic acid methyl ester (1.0 g) and N-hydroxy succinimide (NHS; 0.32 g), followed by 0.36 g of DCC. The solution

was stirred overnight. The solution was then filtered and the filtrate used in the next step.

D. To 1.454 mg BSA in 145 ml of aqueous sodium bicarbonate with stirring was added the filtrate of C. over a time period of 0.5 h at a temperature of about 40° C. After stirring overnight at room temperature, the mixture was centrifuged for 1 h, the supernatant collected and dialyzed 4× for 1 h each time against 4 L of deionized water. The resulting solution having a concentration of about 6 mg/ml was diluted to 3 mg/ml with 2× PBS. Following, 1 ml of 10% NaN<sub>3</sub> was added to the diluted solution and the solution was refrigerated. The solution was warmed to room temperature prior to use.

#### Example 2

##### Preparation of Anti-T<sub>4</sub>-Alkaline Phosphatase Conjugate

A. The alkaline phosphatase was dialyzed against 500 ml of alkaline phosphatase dialysis buffer (76.28 g sodium borate decahydrate in 3.75 ml H<sub>2</sub>O) for 1.5 h with three changes of buffer at room temperature. The dialyzed solution was divided into 400 µl aliquots. 10 µl of 20 mg/ml sulfo-SMCC (sulfosuccinimidyl 4-[maleimidomethyl]) in DMF was added to each aliquot and the mixtures were incubated for about 45 min at room temperature. The reaction mixtures were then dialyzed overnight at 4° C. in modified alkaline phosphatase dialysate buffer (0.1M Tris+5.0 mM MgCl<sub>2</sub>+0.1 mM ZnCl<sub>2</sub>, pH 7.0).

B. After dialyzing IgG-anti-T<sub>4</sub> (2.4 mg/ml) with mouse IgG dialysate buffer (50.0 mM phosphate+1.0 mM EDTA, pH 7.5), and bringing the volume to 1 ml with the dialysate buffer, 10 µl of SATA (N-Succinimidyl S-acetylthioacetate) solution, having a concentration of 1.3 mg SATA/ml DMF, was added and the reaction allowed to proceed for about 30 min at room temperature. The reaction product was dialyzed overnight at 4° C. against 500 ml of IgG dialysate buffer (50.0 mM phosphate+1.0 mM EDTA, pH 7.5).

C. After further dialysis of the modified alkaline phosphatase and the reduced anti-T<sub>4</sub>-IgG, the modified alkaline phosphatase was split equally into two portions and combined with the two portions of reduced anti-T<sub>4</sub> IgG, which has also been divided equally. The two resulting combinations were incubated for one hour at room temperature. 2 µl of NEM solution (0.1 g/ml N-ethylmaleimide in DMF) was added to each portion and the portions were further incubated for 30 min at room temperature. The portions were then combined in a 50 ml centrifuge tube and placed on ice. An equal volume of cold, saturated ammonium sulfate was added dropwise to the now combined portions. The resultant combination was then stirred in a refrigerator overnight.

The precipitate which formed was then centrifuged at 15,000 xg for 30 min. The supernatant was discarded and the precipitate was resuspended in 450 µl of column equilibrium buffer (TBS+5.0 mM MgCl<sub>2</sub>+0.1 mM ZnCl<sub>2</sub>+0.1% NaN<sub>3</sub>). The resuspended precipitate was then run through a column at a flow rate of 0.4 ml/min and aliquot numbers 21-31 (500 µl each) were retained and pooled. 10 mg of BSA were added to the pooled aliquots for every 1.0 ml of pooled aliquot.

#### Example 3

##### Preparation of the Polymerized Polyunsaturated Lipid Layer

The polymerized polyunsaturated lipid layer was produced as follows. First, a glass microscope slide was placed on a copper plate (10 cm×10 cm square and 0.4 cm thick). 2.0 ml of double glass distilled water was applied to one end of the glass slide. The temperature of the water subphase was 30° C. 2.0 µl of a solution of lipid monomers, for example N-(2', 3'-dihydroxy)propyl-3-pentaicosan-10,12-diynamide, was applied to the aqueous surface from a 5.0 µl micropipet at room temperature in two equal aliquots. Enough monomers were applied to achieve a surface area concentration of 1.7 mg/m<sup>2</sup>. Upon evaporation of the solvent, the monomer dried into small visible islands at the water surface. The copper plate was transferred to a preheated hot plate (approximately 200° C. on the hot plate surface). The copper plate, the microscope slide, and the water were heated until the islands of monomer melted and dissolved at the water surface. The copper plate was transferred after 3-5 minutes heating to a prechilled aluminum block embedded in ice. The copper plate, slide and water were allowed to cool to 4° C.

The monolayer was then polymerized with a UV 254 nm short wave lamp (0.06 watts/cm<sup>2</sup>) at a distance of 2 in from the film for a period of 30 sec.

#### Example 4

##### Preparation of the Fluorescence Production Layer

A conjugate of thyroxine and bovine serum albumin was prepared as above, where the ratio of thyroxine to bovine serum albumin was in the range of 1:5 to 1:23. The BSA-T<sub>4</sub> was dissolved to a concentration of 33 mg/ml in Tris-buffered saline pH 7.6 at 25° C. After thorough mixing, an acrylic support was immersed in the buffer mixture and incubated for 1 h at 37° C. The acrylic support was then removed and thoroughly rinsed with distilled water.

The polymerized lipid film was then transferred to the support. Following, the film was treated with TBSt (Tris Buffered Saline containing 0.5% tween 20) by submerging the film, now associated with the solid support, in the TBSt solution and incubated for 10 min at room temperature.

#### Example 5

##### Assay of quantity of T<sub>4</sub> in Blood Plasma

The fluorescence production layer was used to assay the amount of T<sub>4</sub> present in a given sample of plasma in the following manner. First, the fluorescence production layer was placed in contact with the serum sample. Next, a sufficient amount of ANS (8-anilino-1-naphthalenesulphonic acid) was added to the sample so as to release the T<sub>4</sub> which was bound to thyroid binding protein. Addition of ANS resulted in the release of T<sub>4</sub> from the binding protein, resulting in free T<sub>4</sub> analyte. Following, the anti-T<sub>4</sub>-alkaline phosphatase conjugate was added to the sample. The T<sub>4</sub> analyte and the bound BSA-T<sub>4</sub> both competed for binding to the conjugate. The amount of conjugate that bound to the BSA-T<sub>4</sub> was inversely proportional to the amount of T<sub>4</sub> analyte in the plasma. The remaining serum with the unbound T<sub>4</sub> analyte was washed away from the fluorescence production layer. The fluorescence of the film was then



measured with a fluorimeter. Following, a solution of BCIP/NTC (5-bromo-4-chloro-3'-indolyphosphatase/-neotetrazolium chloride) in Tris buffer was washed over the fluorescence production layer. The alkaline phosphatase, now bound to the BSA-T<sub>4</sub> of the fluorescence production layer, acted on the BCIP/NTC to release a dye. The dye quenched the fluorescence production layer and the new fluorescence was measured. By comparing the differences in the measured fluorescence readings with values from a standard curve, the amount of T<sub>4</sub> analyte in the serum sample was determined.

#### Example 6

##### Assay for T<sub>4</sub> in a Serum Sample Using Fluorescence Production Layer in Biocircuits I Instrument

An assay cartridge, roughly the size of credit card, was formed for use in the Biocircuits I instrument. The top half of the cartridge comprised the fluorescence production layer as described above. The bottom half of the assay cartridge was separated into three areas. One area had ANS molecules adsorbed to it, a second had the alkaline-phosphatase conjugate (as prepared above) adsorbed to it, and a third had BCIP/NTC substrate adsorbed to it.

This cartridge was inserted into the Biocircuits I instrument and brought to 37° C. Following, 65 µl of serum, in which the concentration of T<sub>4</sub> was to be determined, was added to the sample port on the instrument. The plasma sample rehydrated the ANS on the cartridge. The rehydrated ANS released T<sub>4</sub> analyte from the thyroid binding proteins to which the T<sub>4</sub> had been bound in the serum. Next, the serum, which now comprised free T<sub>4</sub> analyte, rehydrated the portion of the cartridge with the anti-T<sub>4</sub>-alkaline phosphatase adsorbed to it. T<sub>4</sub> analyte in the serum competed with the bound BSA-T<sub>4</sub> for binding to the rehydrated conjugate. The proportion of conjugate which bound to the BSA-T<sub>4</sub> was inversely proportional to the amount of T<sub>4</sub> analyte which was in the sample. Following sufficient time for competitive binding, the sample was washed away from the cartridge with 250 µl of Tris buffer (pH 9.5).

The fluorescence of the cartridge with bound conjugate was then measured. Following, 280 µl Tris buffer was washed over the cartridge so as to rehydrate the still dry BCIP/NTC substrate. Rehydration brought the substrate into direct contact with the alkaline phosphatase conjugate now bound to the BSA-T<sub>4</sub> on the cartridge. The alkaline phosphatase enzymatically released a blue dye from the rehydrated substrate which quenched the fluorescence of the fluorescence production layer. This enzymatic reaction was allowed to proceed for 10 min. The now quenched fluorescence was measured. By comparing the first fluorescence value with the quenched fluorescence value, the quantity of T<sub>4</sub> present in the original 65 µl sample was determined.

It is evident from the above results, that the subject methodology provides for a rapid and efficient assay for the determination of a wide variety of analytes. The detection system can be readily prepared, without requiring direct conjugation of the member of the specific

binding pair to the lipid. In this manner, the assay is substantially simplified and the formation of the fluorescent lipid layer greatly simplified. A single component lipid film is more reproducible than currently existing two-component systems and the assay procedure is simplified in having fewer molecular building blocks.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

What is claimed is:

1. In a method for detecting the presence of an analyte in a sample wherein the method comprises (a) contacting a sample with a fluorescer and a fluorescence modulation reagent, wherein the fluorescence modulation reagent is a conjugate comprising a first component which specifically binds the analyte or which competes with the analyte for binding to an analyte-specific ligand or first component-specific ligand, and a second component which modulates the fluorescence of the fluorescer, wherein the fluorescence modulation reagent modulates the fluorescence of the fluorescer in relation to the amount of analyte in the sample upon binding the fluorescer, and (b) detecting the modulation of the fluorescence of the fluorescer upon binding; the improvement comprising:
  - said fluorescer comprising
    - (a) a macromolecular analyte-specific or first component-specific ligand non-covalently associated with a polymerized polydiacetylene lipid layer.
  2. A method according to claim 1, wherein said macromolecular ligand is bound to a solid support.
  3. A method according to claim 1, wherein said fluorescence modulation reagent comprises an enzyme and said modulation is the production of an enzyme product capable of quenching said fluorescer.
  4. A method according to claim 1, wherein said macromolecular ligand is an antigen.
  5. A method according to claim 1, wherein said macromolecular ligand is a conjugate of a hapten bound to a protein.
  6. A kit comprising:
    - (a) fluorescent layer comprising:
      - (1) a polymerized polydiacetylene lipid layer;
      - (2) a macromolecular ligand non-covalently associated with said lipid layer; and
    - (c) a conjugate of an enzyme and a member of a specific binding pair; and
    - (d) an enzyme substrate of said enzyme resulting in a product capable of modulating the fluorescence of said fluorescent layer.

\* \* \* \* \*



In re Application of:

Vale et al.

Application No.: 09/502,664

Filed: February 11, 2000

Exhibit C - Page 1



PATENT

Attorney Docket No.: REGEN1500-1

EXHIBIT C

U.S. Patent Number 5,264,557



US005264557A

**United States Patent** [19]

Salomon et al.

[11] Patent Number: **5,264,557**[45] Date of Patent: **Nov. 23, 1993**[54] **POLYPEPTIDE OF A HUMAN  
CRIP-TO-RELATED GENE, CR-3**[75] Inventors: **David S. Salomon, Germantown,  
Md.; Maria G. Persico, Naples, Italy**[73] Assignee: **The United States of America as  
represented by the Department of  
Health and Human Services,  
Washington, D.C.**[21] Appl. No.: **749,001**[22] Filed: **Aug. 23, 1991**[51] Int. Cl.<sup>3</sup> ..... **C07K 3/00; A61K 37/24;  
C12P 21/06; C07H 15/12**[52] U.S. Cl. .... **530/399; 530/350;  
530/387.7; 530/387.9; 435/6; 435/69.1;  
435/7.23; 536/23.51**[58] Field of Search ..... **435/69.1, 6, 7.23;  
530/350, 387, 388, 399; 536/27**[56] **References Cited  
PUBLICATIONS**Ciccodicola et al. (1989), The EMBO Journal, vol. 8,  
No. 7, pp. 1987-1991.*Primary Examiner*—Robert J. Hill, Jr.*Assistant Examiner*—Gian P. Wang*Attorney, Agent, or Firm*—Townsend & Townsend[57] **ABSTRACT**

The present invention relates, in general, to a human CRIPTO-related gene. In particular, the present invention relates to a DNA segment encoding a human CRIPTO-related gene; polypeptides encoded by said DNA segment; recombinant DNA molecules containing the DNA segment; cells containing the recombinant DNA molecule; a method of producing a human CRIP-TO-related polypeptide; a DNA segment encoding a genomic clone of the human CRIPTO gene (CR-1); antibodies specific to CR-3; and a method of measuring the amount of CR-3 in a sample.

**2 Claims, 12 Drawing Sheets**

CR-1 → GCGGGCACTCCCACTGGAGAGTCCAGCTGCCTCTGGCCGCCCCCTCCCTCTCCCGGGCAC

CR-3 → AAGCTTGCGGCCATGTAAGGTAAAGTGACTGATTCTATAGCAATCCAATGTTCTTTGTCTGCCCGTTACATATAACAA  
CTGGCGCGCTCCCGCGTCTTTTCAGGAATTCAGCTCCGCTGGAATTTGCACTTCAAGTCTGGAGCCCCAAGGAACCCCTCTGACCCCTGA

TGTTGTCAATGTTTGATTGAAAATACCTAGCAGGTG

ACTTCTATCTCAGTTTCAAGCTTCTCTAGTCTTCCCCACACACACACCTAGCTCCTCAGGCGGAGAGCACCCCCTTCTTGGCCACCCGGGTATCC

CCCAGGGAGTACGGGGCTCAAAACACCCCTTCTGGAAAAACAAAGGTGGAAGCAAATTTAGGAAGTAAACTTCTGAAATAAAATAAAATATCGA

ATGCCTTGAGACCCATACATTTTCAGGTTTTCTTAATTAAAGCAATTACTTTCCACCACCCCTCCAACCTGGAATCACCACCTTGATTAGAGAAAC

TGATTTTCTTTTCTTTTCTTTTCTTTTCCGAAAAGAGTACCTCTGATCATTTTAGCCTGCAACTAATGATAGAGATATTAGGGCTAGTTAACCACAG

TTTTACAAGACTCCTCTTCCCGCGTGTGGGCCATTGTCATGCTGTCCGGTCCCGCCACCTGAAAGGTCTCCCGCCCGACTGGGGTTTGTGTT

GAAGAAGGAGAAATCCCGGAAAGGCTGAGTCTCCAGCTCAAGGTCAAAACGTTCAAGGCGAAAGCCCTCCAGTTTCCCTTGACGCTTGTCTCT

GCTTCTGCTACGACCTTCTGGGGAACGAATTTCTCATTTTCTTCTTAAATGCCATTTTCGCTTTAGGAGATGAATGTTTCTTTGGCTGTTT

TGGCAATGACTCTGAATTAAGCGATGCTAACGCCCTCTTTCCCCCTAATGTGTTAAAGCTATGGACTGCAGGAAGATGGCCCGCTTCTCTTACAG  
M D C R K M A R F S Y S  
V

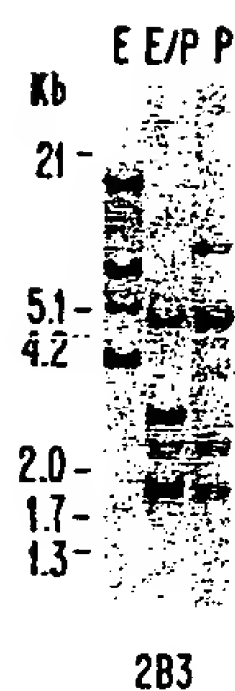


FIG. 1A.

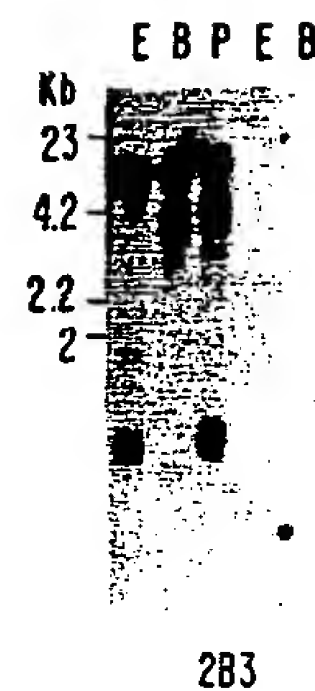
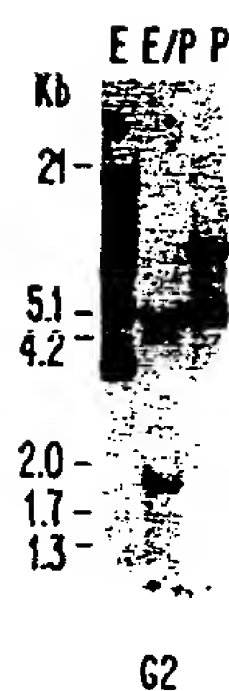


FIG. 1B.

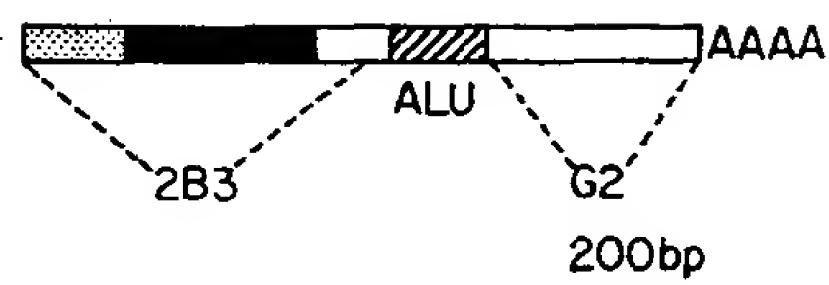


FIG. 1C.

# POLYPEPTIDE OF A HUMAN CRIPTO-RELATED GENE, CR-3

## BACKGROUND OF THE INVENTION

### 1. Field of the Invention

The present invention relates, in general, to a human CRIPTO-related gene. In particular, the present invention relates to a DNA segment encoding a human CRIPTO-related gene; polypeptides encoded by the DNA segment; recombinant DNA molecules containing the DNA segment; cells containing the recombinant DNA molecule; a method of producing a human CRIPTO-related polypeptide; a DNA segment encoding a genomic clone of the human CRIPTO gene (CR-1); antibodies specific to CR-3; and a method of measuring the amount of CR-3 in a sample.

### 2. Background Information

Polypeptide growth factors play a role in stimulating cell proliferation. Their genes are expressed in the developing embryo, in normal adult tissues and in tumor cells (for review see Devel, T. F., *Ann. Rev. Cell Biol.* (1987) 3:443-492; Sporn, M. B. et al., *Nature* (1987) 332:217-219; Whitman, M. et al., *Ann. Rev. Biol.* (1989) 5:93-117). Characterization of these factors and sequencing of their genes have permitted their grouping into a relatively small number of families on the basis of sequence similarities (Mercola, M. et al., *Development* (1988) 108:451-460). One of these is the epidermal growth factor (EGF) family. EGF (Savage, C. R. et al., *J. Biol. Chem.* (1972) 247:7612-7621), transforming growth factor  $\alpha$  (TGF $\alpha$ ) (Derynck, R. et al., *Cell* (1984) 38:287-297) and amphiregulin (AR) (Plowman, G. D. et al., *Mol. Cell Biol.* (1990) 10:1969-1981) share structural similarities including the conservation of six cysteines of the "EGF motif", which in EGF are involved in three disulfide bonds defining the tertiary structure. The presence of "EGF motif" also in developmental genes, such as *Notch* in *Drosophila* (Kidd, S. et al., *Mol. Cell Biol.* (1986) 6:3094-3108) and *lin-12* in *C. elegans* (Greenwald, I., *Cell* (1985) 43:583-590), may imply a novel role for the growth factors of the "EGF family." It has been suggested that they may exert their action on the cell surface during development to mediate cell-cell interactions by recognizing a complementary receptor on another cell.

Previously, the isolation of a human cDNA, referred to as CRIPTO (CR-1) (Ciccodicola, A. et al., *EMBO J.* (1989) 8:1987-1991), encoding a protein of 188 amino acids was described. The central portion of this protein shares structural similarities with the human TGF $\alpha$  (Derynck, R. et al., *Cell* (1984) 38:287-297), human AR (Plowman, G. D. et al., *Mol. Cell Biol.* (1990) 10:1969-1981) and human EGF (Savage, C. R. et al., *J. Biol. Chem.* (1972) 247:7612-7621). Northern blot analysis of a wide variety of tumor and normal cell lines and tissues (e.g., choriocarcinoma, fibroblast, neuroblastoma, HeLa, placenta and testis) has shown that CRIPTO transcripts are detected only in undifferentiated human NTERA-2 clone D1 (NT2/D1) and mouse (F9) teratocarcinoma cells and these disappear after inducing the cells to differentiate with retinoic acid treatment (Ciccodicola, A. et al., *EMBO J.* (1989) 8:1987-1991).

## SUMMARY OF THE INVENTION

It is a general object of this invention to provide a human CRIPTO-related gene (CR-3).

It is a specific object of this invention to provide a DNA segment which encodes a human CRIPTO-related gene (CR-3).

It is a further object of the invention to provide a polypeptide corresponding to a human CRIPTO-related gene (CR-3).

It is another object of the invention to provide a recombinant DNA molecule comprising a vector and a DNA segment encoding a human CRIPTO-related gene (CR-3).

It is a further object of the invention to provide a cell that contains the above-described recombinant molecule.

It is another object of the invention to provide a method of producing a polypeptide encoding a human CRIPTO-related gene (CR-3).

It is a further object of the invention to provide a genomic DNA segment coding for a polypeptide comprising an amino acid sequence corresponding to a human CRIPTO gene (CR-1).

It is a further object of the invention to provide antibodies having binding affinity to a human CRIPTO-related gene (CR-3), or a unique portion thereof and not to CR-1, or a unique portion thereof.

It is a further object of the invention to provide a method of measuring the amount of CR-3 in a sample.

Further objects and advantages of the present invention will be clear from the description that follows.

In one embodiment, the present invention relates to a DNA segment coding for a polypeptide comprising an amino acid sequence corresponding to a human CRIPTO-related gene CR-3.

In another embodiment, the present invention relates to a polypeptide free of proteins with which it is naturally associated and comprising an amino acid sequence corresponding to a human CRIPTO-related gene (CR-3).

In a further embodiment, the present invention relates to a recombinant DNA molecule comprising a vector and a DNA segment that codes for a polypeptide comprising an amino acid sequence corresponding to a human CRIPTO-related gene (CR-3).

In yet another embodiment, the present invention relates to a cell that contains the above-described recombinant DNA molecule.

In a further embodiment, the present invention relates to a method of producing a polypeptide comprising an amino acid sequence corresponding to a human CRIPTO-related gene (CR-3).

In another embodiment, the present invention relates to a genomic DNA segment coding for a polypeptide comprising an amino acid sequence corresponding to a human CRIPTO gene (CR-1).

In yet another embodiment, the present invention relates to an antibody having binding affinity to a human CRIPTO-related gene (CR-3), or a unique portion thereof and not to CR-1, or a unique portion thereof.

In a further embodiment, the present invention relates to a method of measuring the amount of CR-3 in a sample, comprising contacting the sample with the above-described antibodies and measuring the amount of immunocomplexes formed between the antibodies and any CR-3 in the sample.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A-1C CRIPTO-related sequences in human and mouse DNA. FIG. 1A) 10  $\mu$ g of genomic DNA was digested with EcoRI (E), PstI (P) and EcoRI + PstI (E/P), and size-fractionated by agarose gel electrophoresis. Hybridization probes are  $^{32}$ P-nick-translated 2B3 and G2 segments. The molecular weight markers included are HindIII/EcoRI-digested Lambda DNA. FIG. 1B) 10  $\mu$ g of mouse (first three lanes from left) and chicken DNA (fourth and fifth lanes) was digested with PstI (P), BamHI (B) and EcoRI (E). Hybridization probe is  $^{32}$ P-nick-translated 2B3 segment. Electrophoresis, transfer and hybridization were as described below except for washing conditions ( $2\times$  SSC at  $60^\circ$  C). FIG. 1C) Schematic representation of human CRIPTO cDNA. The coding region is indicated by a solid box; AAAA indicates the poly(A) tail. cDNA regions corresponding to 2B3 and G2 probes are indicated.

FIG. 2. Nucleotide sequence of CR-1 and CR-3 genomic DNAs. The sequence of 5763 nucleotides of the CR-1 gene is shown. The nucleotides are numbered from the start codon and the amino acids for CR-1 are shown below. The nucleotide sequence of CR-3 is shown on top of CR-1. Nucleotide changes and deletions ( $\Delta$ ) in the CR-3 sequence are indicated above the CR-1 sequence. The six amino acid changes are indicated below the CR-1 protein sequence. It is to be noted that all the introns of CR-1 are absent in the CR-3 sequence. The boxed motifs are Sp1 binding sites (solid-line boxes), pyrimidine stretches (thin-line boxes) and polyadenylation signals (broken-line box). The vertical arrows indicate the multiple transcription starts. The Alu sequence present in the mRNA is underlined.

FIG. 3. Maps summarizing information obtained from DNA sequencing and restriction mapping of isolated CRIPTO homologous recombinant clones. Top: Physical map of CR-1. Numbered exons are indicated by black boxes for coding region and white boxes for non-coding regions. The hatched box represents the 440 bp upstream of the most common transcription start present also in CR-3. Restriction sites are indicated: EcoRI (E); BamHI (B); PstI (P). Thick lines above the map denote genomic subclones used as probes. Bottom: Physical map of CR-3. Below are represented the genomic region isolated and EcoRI (E), HindIII (H), PstI (P) restriction sites. CR-3 contains all the exons and a polyA tail (AAAA).

FIG. 4A-4C S1 nuclease and primer extension analyses.

FIG. 5A-5C Southern blot analysis using the CR-1-P7 probe.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a human CRIPTO-related gene. This novel human gene (designated CR-3) has been isolated and cloned from a human genomic library using a human CRIPTO cDNA fragment. The CR-3 gene sequence is identical to the human CRIPTO gene sequence with the exception of eight base pair substitutions that give rise to six amino acid changes in the sequence of the protein. The CR-3 human cDNA has been expressed in mammalian COS cells and the recombinantly produced protein can be used to study its biological properties and as an immunogen to generate monospecific antibodies.

CR-3 exhibits partial amino acid sequence homology and a tertiary structure within a 38 amino acid region similar to the EGF supergene family that includes EGF, TGF $\alpha$ , and amphiregulin. Since those peptides are potent mitogens that are involved in regulating the proliferation, differentiation, and transformation of various mesenchymal and epithelial cells, CR-3 like CRIPTO can be expected to be a regulatory molecule that is involved in each of these processes. In addition, expression of CR-3 may serve as a tumor specific marker that may have applicability in the diagnosis, prognosis, and possible treatment of specific types of cancer. In this respect, CR-3 mRNA is expressed in several human colon cancer cell lines and possibly in human colorectal tumors.

In one embodiment, the present invention relates to DNA segment coding for a polypeptide comprising an amino acid sequence corresponding to a human CRIPTO-related gene (CR-3) and allelic and species variation thereof. In a preferred embodiment the DNA segment comprises the sequence shown in SEQ ID NO:4. In another preferred embodiment, the DNA segment encodes the amino acid sequence set forth in SEQ ID NO:5.

In another embodiment, the present invention relates to a polypeptide free of proteins with which it is naturally associated (or bound to a solid support) and comprising an amino acid sequence corresponding to a human CRIPTO-related gene (CR-3) and allelic and species variation thereof. In a preferred embodiment, the polypeptide comprises the amino acid sequence set forth in SEQ ID NO:5.

In another embodiment, the present invention relates to a recombinant DNA molecule comprising a vector (for example plasmid or viral vector) and a DNA segment coding for a polypeptide corresponding to CR-3, as described above. In a preferred embodiment, the encoding segment is present in the vector operably linked to a promoter.

In a further embodiment, the present invention relates to a cell containing the above described recombinant DNA molecule. Suitable host cells include procaryotes (such as bacteria, including *E. coli*) and both lower eucaryotes (for example yeast) and higher eucaryotes (for example, mammalian cells). Introduction of the recombinant molecule into the cell can be effected using methods known in the art.

In another embodiment, the present invention relates to a method of producing a polypeptide having an amino acid sequence corresponding to CR-3 comprising culturing the above-described cell under conditions such that the DNA segment is expressed and the polypeptide thereby produced and isolating the polypeptide.

In a further embodiment, the present invention relates to a DNA segment coding for a polypeptide comprising an amino acid sequence corresponding to a human CRIPTO gene (CR-1) wherein said DNA segment comprises the sequence shown in SEQ ID NO:2. The CR-1 genomic clone can be used in transgenic animals to examine the effects of overexpression of this gene on development and tumorigenicity and to study the regulation of this gene via sequences in the 5'-flanking region that are upstream from the ATG translation initiation codon.

In yet another embodiment, the present invention relates to an antibody having binding affinity to a human CRIPTO-related gene (CR-3), or a unique portion thereof and not to CR-1, or a unique portion

thereof. In one preferred embodiment, CR-3 has the amino acid sequence set forth in SEQ ID NO:5, or allelic or species variation thereof.

Antibodies can be raised to CR-3, or unique portions thereof, in its naturally occurring form and in its recombinant form. Additionally, antibodies can be raised to CR-3 in both its active form and inactive form, the difference being that antibodies to the active CR-3 are more likely to recognize epitopes which are only present in the active CR-3.

CR-3 may be joined to other materials, particularly polypeptides, as fused or covalently joined polypeptides to be used as immunogens. CR-3 or its fragments may be fused or covalently linked to a variety of immunogens, such as keyhole limpet hemocyanin, bovine serum albumin, tetanus toxoid, etc. See for example, Microbiology, Hoeber Medical Division (Harper and Row, 1969), Landsteiner, Specificity of Serological Reactions (Dover Publications, New York, 1962) and Williams et al., Methods in Immunology and Immunochemistry, Vol. 1 (Academic Press, New York, 1967), for descriptions of methods of preparing polyclonal antisera. A typical method involves hyperimmunization of an animal with an antigen. The blood of the animal is then collected shortly after the repeated immunizations and the gamma globulin is isolated.

In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts. Description of techniques for preparing such monoclonal antibodies may be found in Stites et al., editors, Basic and Clinical Immunology, (Lange Medical Publications, Los Altos, CA, Fourth edition) and references cited therein, and in particular in Kohler and Milstein in Nature 256:495-497 (1975), which discusses one method of generating monoclonal antibodies.

Antibodies having binding affinity to a human CRIP-TO-related gene (CR-3), or a unique portion thereof and no to CR-1, or a unique portion thereof can be isolated using screening methods (for example, ELISA assays). Antibodies having binding affinity for CR-3 (with or without binding affinity for CR-1) can be used in immunoassays to detect CR-3.

In a further embodiment, the present invention relates to a method of measuring the amount of CR-3 in a sample, comprising contacting the sample with the above-described antibodies and measuring the amount of immunocomplexes formed between the antibodies and any CR-3 in the sample. Measuring the amount of immunocomplexes formed can be any of those well known in the art, such as RIA, ELISA, and direct and indirect immunoassays.

#### EXAMPLES

The following protocols and experimental details are referenced in the Examples that follow:

##### Southern Blot Analysis and Chromosomal Mapping Panels

All the hybrid cell lines were hamster x human obtained following a published protocol (Davidson, R. D., *Somatic Cell Gen.* (1976) 2:165-176). The hybrid clones were characterized for their human chromosome content (Rocchi, M. et al., *Hum. Gen.* (1986) 74:30-33).

DNA preparation from human peripheral blood lymphocytes and cell lines, restriction enzyme digestion, electrophoresis and Southern blotting were performed using standard techniques (Maniatis, T. et al., *Molecular cloning: A laboratory manual* (1982) Cold Spring Harbor

Laboratory Press, N.Y.). In general, 10 µg of DNA was digested with 40 units of enzyme. Electrophoresis of DNA digests was carried out in agarose gel (0.8%) in TEB buffer (89 mM Tris, 2 mM EDTA, 89 mM boric acid). DNAs were transferred by Southern capillary blot onto nylon membranes ZETABIND (AMF Cuno, Meriden, Conn.), fixed by UV cross-linking and hybridized to 10<sup>6</sup> dpm DNA probes labeled by nick translation (Rigby, P. W. J. et al., *J. Mol. Biol.* (1977) 113:237-251) to a specific activity of about 2×10<sup>8</sup> dpm/µg. Washing was carried out at 65° C. in 2×SSC, 0.2% SDS and subsequently in 0.2×SSC, 0.2% SDS at 65° C.

##### Isolation of CRIPTO Genomic Clones

Genomic clones were isolated from two different human genomic libraries: one obtained by partial MboI digestion of genomic DNA cloned in the BamHI site of the pcos2EMBL Cosmid Vector (Poustka, A. et al., *Proc. Natl. Acad. Sci. USA* (1984), 81:4129-4133), the other obtained by partial MboI digestion of genomic DNA that had been flush ended and cloned into the flush ended XhoI site of Lambda Fix Vector (Stratagene). 5×10<sup>5</sup> cosmids and 10<sup>6</sup> phages were screened using the CRIPTO cDNA fragment 2B3 (see FIG. 1C) by standard techniques (Grunstein, M. et al., *Proc. Natl. Acad. Sci. USA* (1975) 72:3961-3965; Benton, W. et al., *Science* (1977) 196:180-182, respectively). The positive clones were analyzed by restriction mapping and the genomic fragments hybridizing to the human cDNA were subcloned in pUC18 Vector (Yanish-Perron, C. et al., *Gene* (1985) 33:103-109) or in pGEM-1 Vector (Promega). DNA sequencing of the genomic subclones was carried out using the modified dideoxynucleotide chain termination procedure (Hattori M. et al., *Nucl. Acids Res.* (1985) 13:78-13-7827). An oligonucleotide walking strategy was performed using synthetic 17-mer oligonucleotides (Applied Biosystems) deduced from the genomic sequence previously determined.

##### S1 Nuclease Mapping

Total RNA from undifferentiated teratocarcinoma cells NT2/D1 (Andrews, P. W. et al., *Lab. Invest.* (1984) 50:147-162) was isolated by cell lysis in 4M guanidine thiocyanate and sedimentation through 5.7M CsCl (Chirgwin, J. M. et al., *Biochemistry* (1979) 18:5294-5304). Poly(A)+RNA was selected by chromatography on oligo(dT) cellulose (Aviv, H. et al., *Proc. Natl. Acad. Sci. USA* (1972) 69:1408-1412). 5 µg of poly(A)+RNA or 40 µg of total RNA was hybridized with the 320 bp Sau96 fragment of Cr-1-73-H (FIG. 3), <sup>32</sup>P-5'-end labeled, in 20 µl of 40 mM Pipes, pH 7, 0.4M NaCl, 1 mM EDTA, pH 7, and 80% formamide for 16 h at 50° C. Following hybridization, the reaction was diluted 10-fold with S1 nuclease buffer (0.28M NaCl, 0.05M sodium acetate, pH 4.5, 4.5 mM ZnSO and 20 µg/ml single strand DNA). S1 nuclease (1200 units) was added and the reaction mixture was incubated for 2 h at 37° C. The reaction was terminated by the addition of 44 µl of termination buffer (2.5M ammonium acetate and 50 mM EDTA); the DNA:RNA hybrids were extracted with phenol, precipitated with ethanol, resuspended in sequencing dye, heated to 90° C. and resolved on a 6% acrylamide, 7M urea sequencing gel.

##### Primer Extension

For primer extension analysis, a 35 bp synthetic oligonucleotide, ol GP2 (3'-CCCGGTAGAAGGACGT-CAGGTATCGAAATTGTTAA-5') corresponding to



base pairs -9+21 of the first exon was end-labeled using T4 polynucleotide kinase to a specific activity of  $10^8$  cpm/ $\mu$ g of poly(A)+mRNA from NT2/D1 cells (Andrews, P. W. et al., *Lab. Invest.* (1984) 50:147-162) in a 40- $\mu$ l volume containing 10 mM Pipes pH 6.4, 0.4M NaCl, 1 mM EDTA, by heating the reaction mixture for 3 min at 90° C., 2 min at 75° C. and gradual cooling to 42° C. After 14 h at 42° C., the resulting DNA:RNA hybrids were ethanol-precipitated and dissolved in reverse transcription buffer (50 mM Tris HCl pH 8, 0.1M KCl, 10 mM MgCl), in the presence of 500  $\mu$ M deoxynucleotides and 20 units of reverse transcriptase. After 1 h at 42° C., the DNA:RNA hybrids were phenol-extracted, ethanolprecipitated, dissolved in sequencing dye, heated to 90° C. and resolved on a 6% acrylamide, 7M urea sequencing gel.

#### EXAMPLE 1

##### Genomic Complexity of CRIPTO Gene-Related Sequences in Human Chromosomes

The 2020 bp long CRIPTO cDNA previously described (Ciccociola, A. et al., *EMBO J.* (1989) 8:1987-1991) contains an open reading frame of 564 bp, a 245 bp long 5' untranslated region, and a 1209 bp long 3' untranslated region that includes an Alu sequence element.

As a first approach to characterize the genomic organization of the gene encoding the CRIPTO protein, Southern blot analyses were carried out. The two cDNA fragments, 2B3 and G2 (FIG. 1C), used as probes, hybridized to several genomic restriction fragments (FIG. 1A). The 2B3 probe, used to analyze by Southern blot the genomic DNA of mouse and chicken, hybridized to several bands in the lanes containing mouse DNA (FIG. 1B, first three lanes), whereas no hybridization was seen with chicken DNA (FIG. 1B fourth and fifth lanes).

#### EXAMPLE 2

##### Isolation and Characterization of CRIPTO Human Genomic Clones

To better understand the nature of the CRIPTO gene-related sequences, a human genomic library (Poustka, A. et al., *Proc. Natl. Acad. Sci. USA* (1984), 81:4129-4133) was screened using CRIPTO fragment 2B3 as a probe and 34 positive cosmid clones were isolated. EcoRI restriction analysis of 10 of the isolated clones revealed only 3 different restriction patterns in the inserts.

The isolated clones were hybridized to a synthetic oligonucleotide (G1) corresponding to nucleotides -91 to -110 of the 5' non-coding region of CRIPTO cDNA (Ciccociola, A. et al., *EMBO J.* (1989) 8:1987-1991 and FIG. 2), with the intention of isolating the complete gene and discarding possible incomplete pseudogenes. A positive 800 bp PstI/EcoRI fragment (CR-1-P7) was identified in the CR-1 cosmid clone (FIG. 3 top).

DNA sequencing analysis revealed that clone CR-1 includes an intact structural gene encoding the entire human CRIPTO protein. The CRIPTO coding sequence is encoded by six exons spanning a 4.8 kb long DNA interval (FIG. 3 top). The nucleotide sequences at the exon-intron boundaries were established by DNA sequence comparison of cDNA and genomic subclones. The 5' donor and 3' acceptor splice sites in each of the

five introns conform to the GT.....AG rule and agree with the consensus sequence compiled for the exon-intron boundaries (Mount, S. M., *Nucl. Acids Res.*, (1982) 10:459-472) except for the acceptor sequence of the second and third introns (FIG. 2). Exon 1 is 281 bp in length and contains the initiator methionine. The other exons range in size from 52 to 1329 bp. The most 3' exon, 1329 bp in length, contains 118 bp of coding sequence and all of the 3' untranslated region (3' UT), which is 1209 nucleotides long (FIG. 2). The EGF-like domain exhibited by the CRIPTO protein (Ciccociola et al. 1989) is encoded by exon 4.

A combination of S1 nuclease mapping and primer extension analyses was used to characterize the CR-1 transcription products.

Since the CRIPTO gene was found to be expressed in an undifferentiated human teratocarcinoma cell line (NT2/D1) (Ciccociola et al. 1989), poly (A)+RNA isolated from cultured NT2/D1 cells was used. The probe used for S1 nuclease mapping was a double-strand DNA fragment encompassing nucleotides -302 to +18 of the genomic sequence and was labeled with  $^{32}$ P at the 5' end (FIG. 4C). Five major S1 nuclease-protected fragments (FIG. 4A) mapping between positions -180 to -253 of the genomic sequence were observed (FIG. 4C).

The primer extension assay, performed with o1 Gp2 (FIG. 4C) confirmed the five major products corresponding in length to the transcripts predicted by S1 analysis (FIG. 4B). It should be noted that other bands are seen in primer extension experiments probably due to both minor RNA species and early termination of the reverse transcriptase reaction.

#### EXAMPLE 3

##### Chromosome Mapping with Somatic Cell Hybrid Panel

A chromosome mapping panel was used to assign the CR-1 gene to human chromosomes. A 1.5 kb long PstI fragment derived from CR-1 (CR-1-P3, FIG. 3 top) was used to probe a Southern blot of TaqI-digested genomic DNAs prepared from 23 hamster human somatic cell hybrids (Table I). Under conditions of high stringency one human specific genomic fragment of 4.5 kb hybridized to the probe. The presence of the 4.5 kb fragment could be clearly distinguished in the DNA of the hybrid cell lines containing chromosome 3 (Table I).

When the EcoRI-PstI fragment (CR-1-P7) containing 800 bp upstream of the translation initiation (see FIG. 3 top) was used to probe the same Southern blot described previously and shown in FIG. 1, hybridization to two fragments was seen (e.g., in the lane containing human DNA digested with EcoRI and PstI (FIG. 5A), the 0.8 kb band corresponds to the genomic sequence CR-1). This indicated that the 5' region of the CR-1 gene was present in two copies in the human genome. When the CR-1-P7 fragment was used to probe the above-mentioned hamster-human somatic cell hybrid panel, it was possible to obtain the segregation of the two sequences (FIG. 5B). Because of the hybridization pattern summarized in Table I and, in particular, the pattern obtained using the hybrid cell lines containing portions of the X chromosome already described (Rocchi et al. 1986), the second genomic copy can be assigned to the Xq21-22 region.

TABLE 1

Segregation of CRIPTO-related sequences in human/hamster hybrids			
Cell lines	Chromosomes present	CR-1	Cr-3
HY.19.16T3D	Xq-, 10, 12, 13, 14, 15, 18, 20	-	-
HY.22AZA1	t(X;X) <sup>a</sup> , 5, 12, 14, 17, 18, 19	-	+
HY.31.24E	X, 5, 8, 11, 12, 14, 21	-	+
HY.36.1	X, 8, 11, 19	-	+
HY.60A	X, 5, 6, 8, 13, 14, 18, 20	-	+
HY.70B1A	t(X;21) <sup>b</sup> , 6, 15, 16	-	-
HY.70B2	t(X;21) <sup>b</sup> , 6, 13, 15, 16	-	-
HY.75E1	X, 5, 9, 12	-	+
HY.94A	X, 6, 7, 8, 16, 22	-	+
HY.94BT1	t(X;Y) <sup>c</sup> , 4, 7, 9, 11, 12, 20	-	+
HY.95A1	X, 3, 5, 10, 11, 14	+	+
HY.95B	X, 4, 6, 7, 14, 18, 22	-	+
HY.95S	X, 2, 3, 13, 21	+	+
HY.112F7	t(X;11) <sup>d</sup> , 3, 4, 8, 10, 20	+	+
RJ.369.1T2	13, 22	-	-
Y.173.5CT3	Xi, 1, 3, 4, 6, 8, 11, 12, 14, 15, 18, 21, 22	+	+
YC2T1	X, 1, 11, 12, 14, 18, 19, 20	-	+
HY.136C	X	-	+
Y.X6.8B2	t(X;6) <sup>e</sup> , 1, 3, 5, 12, 13, 14, 15, 17, 21, 22	+	+
Y.162AZA	t(X; hamster) <sup>f</sup>	-	+
HY.87Z4	t(X;11) <sup>g</sup> , 1, 2, 4, 5, 6, 12, 15, 20	-	-
HY.85D30T2	t(X;1) <sup>h</sup> , 2, 3, 8, 11, 13, 18, 21	+	+
HY.84T2	Y	-	-
Chromosome assignment		3	Xq21-Xq22
Number concordant +		6	17
Number concordant -		17	6
Number discordant		0	0

Note: + and - indicate, respectively, presence or absence of CR-1 and CR-3 sequences

<sup>a</sup>Xqter -> Xq21::Xp22.3 -> Xqter

<sup>b</sup>Xqter -> Xq22::21p13 -> 21qter

<sup>c</sup>Xqter -> Xp22.3::Yp -> Yqter

<sup>d</sup>Xqter -> Xq11.1::p11.2 -> 11q11

<sup>e</sup>Xqter -> Xq21.3::6q27 -> 6pter

<sup>f</sup>Xpter -> Xq27.3::hamster

<sup>g</sup>Xqter -> Xq26::11q23 -> 11pter

## EXAMPLE 4

## Isolation and Characterization of a Second Genomic CRIPTO-Related Sequence

A genomic library was screened to isolate the genomic clones containing the 5' cDNA non-coding region using as probe the labeled CR-1-P7 DNA fragment (FIG. 3). Only two different classes of recombinant phages were found exhibiting the restriction pattern expected from the Southern blot (FIG. 5A-5B).

The restriction map of clone CR-3 is shown in FIG. 3 bottom. To investigate whether the CRIPTO related genomic sequences from recombinant lambda CR-3 clones encode a complete CRIPTO protein, the nucleotide sequence of a 2688 bp fragment hybridizing to 2B3 and G2 was determined and compared this sequence with that of cDNA (FIGS. 2 and 3 bottom).

Analysis of the nucleotide sequence of CR-3 revealed that this clone includes a complete CRIPTO cDNA lacking introns and containing a poly(A) tract at the 3'

end. Seven single base pair substitutions are observed in the coding region (FIG. 2) and six of these give rise to amino acid changes. The 3' non-coding sequence is less similar (97% identical) to CR-1. Most of the base changes, deletions and insertions fall within the inverted Alu sequence. The unusual polyA addition site AGTAAA present in the CR-1 gene is conserved also in CR-3. The similarity between CR-1 and CR-3 extends for 697 nucleotides upstream of the initiator AUG where it is possible to observe 7 base pair substitutions and 6 nucleotide deletions.

All publications mentioned hereinabove are hereby incorporated in their entirety by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.

## SEQUENCE LISTING

## ( 1 ) GENERAL INFORMATION:

( 1.1 ) NUMBER OF SEQUENCES: 4

## ( 2 ) INFORMATION FOR SEQ ID NO:1:

## ( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 35 base pairs

( B ) TYPE: nucleic acid

( C ) STRANDEDNESS: single

( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:1:



-continued

AATTGTTAAA GCTATGGA CT CAGGAAGAT GCGCC

33

## ( 2 ) INFORMATION FOR SEQ ID NO:2:

## ( 1 ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 5761 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: double
- ( D ) TOPOLOGY: linear

## ( 1 ) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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GCCGGCACTC CCACTGGAGA GTCCAGCTG CCTCTGGCCG CCCCTCCCCT CTCCCGGGCA      60
CCTGGCGCCG CTCCCGCGTC CTTTCAGGAA TTCACGTCCG CCTGGAATTT GCACTTCAAG      120
TCTGGAGCCC CCAAGGAACC CCTCCTGACC CTGAAGTTCT ATCTCAGTTT CAAGCTTCCT      180
AGTCTTCCCC ACACACACAC ACCTAGCTCC TCAGGCGGAG AGCACCCCTT TCTTGGCCAC      240
CCGGGTATCC CCCAGGGAGT ACGGGGCTCA AAACACCCTT CTGGAAAAAA CAAAGGTGGA      300
AGCAAATTTT AGGAAGTAAA ACTTCTGAAA TAAAAATAAA TATCGAATGC CTTGAGACCC      360
ATACATTTTC AGGTTTTTCT AATTAAAGCA ATTACTTTCC ACCACCCCTC CAACCTGGAA      420
TCACCAACCT GATTAGAGAA ACTGATTTTT CTTTTTTCTT TTTTTTTCCC GAAAAGAGTA      480
CCTCTGATCA TTTTAGCCTG CAACTAATGA TAGAGATATT AGGGCTAGTT AACCACAGTT      540
TTACAAGACT CCTCTTCCCG CGTGTGGGCC ATTGTCATGC TGTCGGTCCC GCCCACCTGA      600
AAGGTCTCCC CGCCCCGACT GGGGTTTTGT GTTGAAGAAG GAGAATCCCC GGAAGGCTG      660
AGTCTCCAGC TCAAGGTCAA AACGTCCAAG GCCGAAAAGC CTCCAGTTTC CCCTGGACGC      720
CTTGCTCCTG CTTCTGCTAC GACCTTCTGG GGAAAACGAA TTTCTCATTT TCTTCTTAAA      780
TTGCCATTTT CGCTTTAGGA GATGAATGTT TTCTTTTGGC TGTTTTGGCA ATGACTCTGA      840
ATTAAGCGA TGCTAACGCC TCTTTTCCCC CTAATTGTTA AAAGCTATGG ACTGCAGGAA      900
GATGGCCCGC TTCTCTTACA GGTATGAGCT AATCTTAGAA TAGTGAAGTT TTTTTOATTG      960
CTAGAGATTG CCAGCTTAGG AAGTAATGTT CTACACTGTC ATTTGATTTT TCTCCTTGCT     1020
CAAGCCTTAA AAGAGCTGCC AACCGACTGC TGTTTTTCCT GAAAGACCTG GAATTTTACA     1080
TGTTACTTC TAACTTTGCC ATTGGCTTTT AACATTTTCG TGTTAATGTT AATTTTCATT     1140
TTATGTTAAT GACTCTGCCT ATGAAGTAGT GTTCTTTTAC TTCTTGATCA AATAAAGGTC     1200
AGTACTACAA CCAAATTTAA ATCTTCCGAA AAGATTAAAG GTATAAGCAG ATTCAATACT     1260
TGGCAAAACT ATTAAGATAA TAGCAAAAAA AAAAAAAAAA CCCACATTTT TTACCTAAAA     1320
ACCTTTTAAG TGATTGGTTA AAATAGTTTG GCCGGGTGCG GTGGCTCACG CCTGTAATCC     1380
TAGCACTTTG GGAGGCAGAG GCGGGTGGAT CACTGAGGTC AGGAGACCAG CCTGGCCAAC     1440
ATGGCAAAAC CCCGTCTCTA TAAAAAATAC AAAAATTAGC CAAGCATGGT GCGGGGCACC     1500
TGTAATCCCA GCTACTCTGG AGGCTGAGGC AGGAGAATTG CTTGAACTGG GGAGGGGAAG     1560
CAGTGAGCCG AGATCGCACC ATTGCACTCC AGCCTGGGTG AAAAACCAGAA ACTCCCTCTC     1620
AAAAATAAAT AAATAAATAC AGTAGTTTGT AAAATGATTC ATCGGTAACA TGGGATGCAG     1680
CTATTTTATA ATCCTTATAT GAAAATTGTA TGCAGGGGAA AACATGTGAA ATAGAAGATA     1740
AAAGACATAT ACCTACTTAA AATTAGGTAC TTATGTGAGG ACAGGGCCTA AGAAATAATA     1800
ATATATATTA AAAAGACTTG GATATTGGTG ACTTTTTTTC AACATTTTTC TTTGTTACAT     1860
GAATTAGCCA TTAATAAAAG AAAGATGGTG CTCTACAATT TCTTTTCAGT GATCTGTGGT     1920
CTTGTCCTTG TGATGAGAGG ACCTGGGTGT TAACTTGTA GGTTTTATTT CCTTTGTTTG     1980
GCTAACTCAT GTTTGACTTC CTCTTCTAG TGTGATTTGG ATCATGGCCA TTTCTAAAGT     2040

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CTTTGAACTG	GGATTAGTTG	CCGGTGAGAG	ACCTTTTGT	TCTTTTGATC	ACTCTCAATT	2100
TTATGTGCC	TAAAATACAG	ACTCCATGAA	TGATTTGTC	GTAAAGGGCT	GGGCCATCAG	2160
GAATTTGCTC	GTCCATCTCG	GGGATACCTG	GCCTTCAGAG	ATGACAGCAT	TTGGCCCCAG	2220
GAGGAGCCTG	CAATTCGCCC	TCGGTCTTCC	CAGCGTGTGC	CGCCCATGGG	GATACAGCAC	2280
AGTAAGAACT	GCCTGACTTC	GATGCTTCTG	CCGTGGCCCT	TCATGTGTCT	CCTGACTATC	2340
TTTCCAACAC	TCTTTCACCT	AAAAGGGCAC	CTGGTTCTGG	AACTGTGCAG	GTGCTGGAAT	2400
GCTTTGGTTT	TGGAAGTGAG	ACAAGGATTG	TGTATTTTAC	TTCCCTAGAG	TGCAGTTTCC	2460
TCCCCTGAGT	CCACTTCACA	CTGGGAACCC	AGAACCACCA	CTGGCCTATG	CATGAAAATG	2520
ACTTCTCTGC	TCAAAGGCAC	AGAGTCTTAC	TCTGATACAA	CACATTGGTG	TTGTATTAAC	2580
CTTCGCTTAC	AGGAATTGCC	CTTGCACTTT	TCCATCCCTA	CACCTCAGTC	ATTCTGTTCT	2640
TACCTTTCAA	GGTAAGGAGC	TAAACAGAAC	CTGCTGCCTG	AATGGGGGAA	CCTGCATGCT	2700
GGGGTCCTTT	TGTGCCTGCC	CTCCCTCCTT	CTACGGACGG	AACTGTGAGC	ACGATGTGCG	2760
CAAGAGTAA	GCAATTCAGA	GGGGCGGGGA	GCCGTGGAGA	GGAGAGAGAA	AGGGAAGTGG	2820
AAATTTTACA	CCCAAGCTAT	CGCAGCTTAC	CTGTTTATTG	TCAGGAAGTG	TGGGTCTGTG	2880
CCCCATGACA	CCTGGCTGCC	GAAGAAGTGT	TCCCTGTGTA	AATGCTGGCA	CGGTCAAGCTC	2940
CGCTGCTTTC	CTCAGGCATT	TCTACCCGGC	TGTGGTAAGC	GGAGGTTCTC	CTCTTTCTTT	3000
TGCCCTTTGA	AGTTACGTAG	TTGCCTTGGG	GGGTGCTTAG	TTAGCAGGCT	CTCCTTGATC	3060
CTCTTGCTTT	GCTAGAGCCT	GGCAGCCAAA	GTTCTGCTTA	TAAAAGCATC	GCAGACTCCT	3120
GATGAGATAG	TTGCCTTGGC	CTCTTTGATA	TTTATTTTCT	CGGGAACCTG	GCTAGTCCTG	3180
CTGCCTTTCA	GATAGAGATG	TATTTCAAAT	CTATTTGACA	TTTTATGGTC	TGAACCTCTA	3240
TTGAGGAAAA	TAAACAAGTC	TCGGTCTCTT	GTAAACCAA	GAGATGTTCT	CTGGTGTTC	3300
TTTCCTTTGG	GTAGGGGGGA	CCCAAACCAG	GATGGGCAGC	TCATTTAGAG	CCCACCCTGA	3360
CGACAAATTC	TATCAGAGGC	TTGGCCCCCT	GCTAGTCCTT	TAGAAACTTC	CAGAGTCCTA	3420
AAAGTCCCTG	GTAACCCCTT	CCCCATACCT	TACCATGACT	GGTCACAGAA	CCCTTACCAT	3480
GACTGGTCAC	AGAACCCTTT	CACCTTCTTG	ATTTTTTACT	GATTTGAGGA	ATACAATGAA	3540
AAGAAGGGCA	GCACCTGGAG	AGGAAAAGAG	GCGACAGTCC	TCTCTCCACC	CTAGCCTGAG	3600
CCAGGTTTCT	AGGGCCCCCC	AAATTCAGAG	ACCTATTATA	GTTCTGGGCC	TTGGAAGTGT	3660
AGAAATGGAA	AATATTCAAG	CCCAGGAAGT	AAATGAAAGC	AAACATTTCA	CTGAGAACAG	3720
GAAGGAATTC	CCCAATCCAG	ACAGGGATTG	TGTCTTTGCC	ATTTGCATCC	TGGGTOTCAG	3780
GCTCAGGATA	GGTGTTTGAT	AAGTGTGGGT	TGGGTGATTG	GATGTGTAGG	GAACATTTGC	3840
TCTTCCTGGA	ACATGGGGCC	CAAGTCAGAA	TCTAACCAG	GTTGTGCTCA	TTCTTGCAAG	3900
TGAAGGCATC	ACCACTGGGC	TAGGTTCCAG	GTGTGAGTGT	CCTGAGAAGA	GCAGGTTTAC	3960
AGTAGCGTAT	AGATATGCCA	CATTTGTGGG	CAGCAGGATG	AACTGCCAGA	GAGGTTTGCT	4020
TTAATGACCA	AGCATCCCTA	CCTTCCAGAT	GGCCTTGTTA	TGGATGAGCA	CCTCGTGGCT	4080
TCCAGGACTC	CAGAACTACC	ACCGTCTGCA	CGTACTACCA	CTTTTATGCT	AGTTGGCATC	4140
TGCCTTTCTA	TACAAAGCTA	CTATTAATCG	ACATTGACCT	ATTTCCAGAA	ATACAATTTT	4200
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TGATCATTTG	TAGTTGCCTT	AAAATAATGA	ATACAATTTT	CAAAATGGTC	TCTAACATTT	4320
CCTTACAGAA	CTACTTCTTA	CTTCTTTGCC	CTGCCCTCTC	CCAAAAAACT	ACTTCTTTTT	4380
TCAAAAGAAA	GTCAGCCATA	TCTCCATTGT	GCCTAAGTCC	AGTGTTTCTT	TTTTTTTTTT	4440
TTTTTGAGAC	GGACTCTCAC	TCTGTCACCC	AGGCTGGACT	GCAATGACGC	GATCTTGGTT	4500

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CACTGCAACC	TCCGCATCCG	GGGTTCAAGC	CATTCTCCTG	CCTAAGCCTC	CCAAGTAACT	4560
GGGATTACAG	GCATGTGTCA	CCATGCCCAG	CTAATTTTTT	TGTATTTTTA	GTAGAGATGG	4620
GGGTTTCACC	ATATTGCCCA	GTCTGGTCTC	GAAGTCCTGA	CCTTGTGATC	CACTCGCCTC	4680
AGCCTCTCGA	AGTGCTGAGA	TTACACACGT	GAGCAACTGT	GCAAGGCCTG	GTGTTTCTTG	4740
ATACATGTAA	TTCTACCAAG	GTCTTCTTAA	TATGTTCTTT	TAAATGATTG	AATTATATGT	4800
TCAGATTATT	GGAGACTAAT	TCTAATGTGG	ACCTTAGAAT	ACAGTTTGA	GTAGAGTTGA	4860
TCAAAATCAA	TTAAAATAGT	CTCTTTAAAA	GGAAAGAAAA	CATCTTTAAG	GGGAGGAACC	4920
AGAGTGCTGA	AOGAATOGAA	CTCCATCTCC	GTGTGTGCAG	GGAGACTGGG	TAGGAAAGAG	4980
GAAGCAAATA	GAAGAGAGAG	GTTGAAAAAC	AAAATGGGTT	ACTTGATTGG	TGATTAGGTG	5040
GTGOTAGAGA	AGCAAGTAAA	AAGGCTAAAT	GGAAAGGCCA	GTTTCCATCA	TCTATAGAAA	5100
GCTATATAAG	ACAAGAAGTC	CCCTTTTTTT	CCCAAAGGCA	TTATAAAAAG	AATGAAGCCT	5160
CCTTAGAAAA	AAAATTATAC	CTCAATGTCC	CCAACAAGAT	TGCTTAATAA	ATTGTGTTTC	5220
CTCCAAGCTA	TTCAATTCTT	TTAACTGTTG	TAGAAGACAA	AATGTTTACA	ATATATTTAG	5280
TYGTAAACCA	AGTGATCAAA	CTACATATTG	TAAAGCCCAT	TTTTAAAATA	CATTGTATAT	5340
ATGTGTATGC	ACAGTAAAAA	TGGAACTAT	ATTGACCTAA	ATGTGAAGTG	GTTATTTCTA	5400
GGTGGTGAGG	TGCTTTATGG	TGGTGGGTTT	TYGCTCTTGA	TGCCCTTTTT	GCATTTTCCA	5460
AAGTACCATG	GTGAGGATGT	GTTATATCTT	TYCCAGGGTC	CTAAAAGTCC	CTGGCAAGTC	5520
CCTCCCCATA	CCCTACCATG	ACTGGTCACA	GAACCTTTTC	ACCTTATTGA	TTTGTACTGA	5580
TTTCATATGG	AATATGGCAA	CTACATCTGG	CTCAAAACAA	AGGAAACCAG	AAGAGCCAAG	5640
TCCCAGGTGA	GTGCTCAGTT	CTGTTTCTAG	CTTTGACGTG	TGTGTTCTTC	TGTGAAGGAC	5700
AAAATTTGCT	TCTATTATTT	AGGTACCATA	ATTTGTOTTT	TTCCAAATTA	ATTCCCTGCA	5760
G						5761

## ( 2 ) INFORMATION FOR SEQ ID NO:3:

## ( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 188 amino acids

( B ) TYPE: amino acid

( D ) TOPOLOGY: linear

## ( ii ) MOLECULE TYPE: protein

## ( iii ) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met	Asp	Cys	Arg	Lys	Met	Ala	Arg	Phe	Ser	Tyr	Ser	Val	Ile	Trp	Ile
1				5					10					15	
Met	Ala	Ile	Ser	Lys	Val	Phe	Glu	Leu	Gly	Leu	Val	Ala	Gly	Leu	Gly
			20					25					30		
His	Gln	Glu	Phe	Ala	Arg	Pro	Ser	Arg	Gly	Tyr	Leu	Ala	Phe	Arg	Asp
		35					40					45			
Asp	Ser	Ile	Trp	Pro	Gln	Glu	Glu	Pro	Ala	Ile	Arg	Pro	Arg	Ser	Ser
	50				55						60				
Gln	Arg	Val	Pro	Pro	Met	Gly	Ile	Gln	His	Ser	Lys	Glu	Leu	Asn	Arg
65					70				75					80	
Thr	Cys	Cys	Leu	Asn	Gly	Gly	Thr	Cys	Met	Leu	Gly	Ser	Phe	Cys	Ala
			85					90						95	
Cys	Pro	Pro	Ser	Phe	Tyr	Gly	Arg	Asn	Cys	Glu	His	Asp	Val	Arg	Lys
			100				105						110		
Glu	Asn	Cys	Gly	Ser	Val	Pro	His	Asp	Thr	Trp	Leu	Pro	Lys	Lys	Cys
	115					120					125				
Ser	Leu	Cys	Lys	Cys	Trp	His	Gly	Gln	Leu	Arg	Cys	Phe	Pro	Gln	Ala
	130					135					140				

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Phe Leu Pro Gly Cys Asp Gly Leu Val Met Asp Glu His Leu Val Ala  
 145 150 155 160  
 Ser Arg Thr Pro Glu Leu Pro Pro Ser Ala Arg Thr Thr Thr Phe Met  
 165 170 175  
 Leu Val Gly Ile Cys Leu Ser Ile Glu Ser Tyr Tyr  
 180 185

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2675 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

## (ix) FEATURE:

- (A) NAME/KEY: CDS  
 (B) LOCATION: 809..1372

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AAGCTTGCGC GCCATGTAAG GTAAAGTGAC TGATTCTATA GCAATCCAAT TGTTCCTTTG 60  
 TCTGCCCGTT TACATATAAC AATGTTGTCA ATGTTTGATT GAAAATACCT AGCAGGTGAC 120  
 ACACACACAC CTAGCTCCTC AGGCGGAGAG CACCCCTTTC TTGGCCACCC GGGTATCCCC 180  
 CAGGGAGTAC GGGGCTCAAA ACACCCTTTT GGAGAACAAG GTGGAAOCAA ATTTCAOGAA 240  
 GTAAAACTTC CGAAATAAAA TAAAATATCG AATGCCTTGA GACCCATACA TTTTCAGGTT 300  
 TTCCTAATTA AAGCAATTAC TTTCACCAC CCCTCCAACC TGGAATCACC AACTTGGTTA 360  
 GAGAAACTGA TTTTCTTTT TTCTTTTTT TTCCCAAAAG AGTACATCTG ATCATTTTAG 420  
 CCTGCAACTA ATGATAGAGA TATTAGGGCT AGTTAACCAC AGTTTACAA GACTCCTCTC 480  
 CCGCGTGTGG GCCATTGTCA TGCTGTGGT CCCGCCACC TGAAAGGTCT CCCCOCCTCC 540  
 ACTGGGGTTT GTTGTGAAG AAGGAGAATC CCCGGAAAGG CTGAGTCTCC AGCTCAAGGT 600  
 CAAAACGTCC AAGGCCGAAA GCCCTCCAGT TTCCCTGGA CACCTTGCTC CTGCTTCTGC 660  
 TACGACCTTC TGGGAACGCG AATTTCTCAT TTTCTTCTTA AATTGCCATT TTCGCTTTAG 720  
 GAGATGAATG TTTTCCTTTG GCTGTTTTGG CAATGACTCT GAATTAAAGC GATGCTAACG 780  
 CCTCTTTTCC CCCTAATTGT TAAAAGCT ATG GAC TGC AAG AAG ATG GTC CGC 832  
 Met Asp Cys Arg Lys Met Val Arg  
 1 5  
 TTC TCT TAC AGT GTG ATT TGG ATC ATG GCC ATT TCT AAA GCC TTT GAA 880  
 Phe Ser Tyr Ser Val Ile Trp Ile Met Ala Ile Ser Lys Ala Phe Glu  
 10 15 20  
 CTG GGA TTA GTT GCC GGG CTG GGC CAT CAA GAA TTT GCT CGT CCA TCT 928  
 Leu Gly Leu Val Ala Gly Leu Gly His Gln Glu Phe Ala Arg Pro Ser  
 25 30 35 40  
 CGG GGA GAC CTG GCC TTC AGA GAT GAC AGC ATT TGG CCC CAG GAG GAG 976  
 Arg Gly Asp Leu Ala Phe Arg Asp Asp Ser Ile Trp Pro Gln Glu Glu  
 45 50 55  
 CCT GCA ATT CGG CCT CGG TCT TCC CAG CGT GTG CTG CCC ATG GGA ATA 1024  
 Pro Ala Ile Arg Pro Arg Ser Ser Gln Arg Val Leu Pro Met Gly Ile  
 60 65 70  
 CAG CAC AGT AAG GAG CTA AAC AGA ACC TGC TGC CTG AAT GAG GGA ACC 1072  
 Gln His Ser Lys Glu Leu Asn Arg Thr Cys Cys Leu Asn Glu Gly Thr  
 75 80 85  
 TGC ATG CTG GGG TCC TTT TGT GCC TGC CCT CCC TCC TTC TAC GGA CGG 1120  
 Cys Met Leu Gly Ser Phe Cys Ala Cys Pro Pro Ser Phe Tyr Gly Arg  
 90 95 100  
 AAC TGT GAG CAC GAT GTG CGC AAA GAG AAC TGT GGG TCT GTG CCC CAT 1168  
 Asn Cys Glu His Asp Val Arg Lys Glu Asn Cys Gly Ser Val Pro His

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105	110	115	120	
GAC ACC TGG CTG CCC AAG AAG TGT TCC CTG TGT AAA TGC TGG CAC GGT				1216
Asp Thr Trp Leu Pro Lys Lys Cys Ser Leu Cys Lys Cys Trp His Gly	125	130	135	
CAG CTC CGC TGC TTT CCT CAG GCA TTT CTA CCC GGC TGT GAT GGC CTT				1264
Gln Leu Arg Cys Phe Pro Gln Ala Phe Leu Pro Gly Cys Asp Gly Leu	140	145	150	
GTG ATG GAT GAG CAC CTC GTG GCT TCC AGG ACT CCA GAA CTA CCA CCG				1312
Val Met Asp Glu His Leu Val Ala Ser Arg Thr Pro Glu Leu Pro Pro	155	160	165	
TCT GCA CGT ACT ACC ACT TTT ATG CTA GCT GGC ATC TGC CTT TCT ATA				1360
Ser Ala Arg Thr Thr Thr Phe Met Leu Ala Gly Ile Cys Leu Ser Ile	170	175	180	
CAA AGC TAC TAT TAATCGACAT TGACCTAITT CCAGAAATAC AATTTTAGAT				1412
Gln Ser Tyr Tyr	185			
ATTATGCAAA TTTCATGACC CGTAAAGGCT GCTGCTACAA TGTCCTAACT GAAAGATGAT				1472
CATTTGTAGT TGCCTTAAAA TAATGAATAC AATTTCCAAA ACGGTCTCTA ACATTTTCCTT				1532
ACAGAACTAA CTACTTCTTA CCTCTTTGCC CTGCCCTCTC CCAAAAAACT ACTTCTTTTT				1592
TCAAAAGAAA GTCAGCCATA TCTCCATTGT GCCCAAGTCC AGTGTTCCTT TTTTTTTTTT				1652
GAGACGGACT CTCACTCTGT CACCCAGGCT GGACTGCAAT GACGCGATCT TGGTTCACCG				1712
CAACCTCCGC ATCCGGGGTT CAAGCCATTC TCCTGCCTCA GCCTCCCAAG TAGCTGGGAT				1772
TACAGGCATG TGTCAACATG CCGGCTAATT TTTTGTATT TTTAGTAGAG ACGGGGGTTT				1832
CACCATATTG GCCAGTCTGG TCTCGAACTC TGACCTTGTG ATCCATCGCT CGCCTCTCAA				1892
GTGCTGAGAT TACACACGTG AGCAACTGTG CAAGGCCTGG TGTTCCTTGA TACATGTAAT				1952
TCTACCAAGG TCTTCTTAAT ATGTTCTTTT AAATGATTGA ATTATACACT CAGATTATTG				2012
GAGACTAAGT CTAATGTGGA CCTTAGAATA CAGTTTTGAG TAGAGTTGAT CAAAATCAAT				2072
TAAATAGTC TCTTTAAAAG GAAAGAAAAC ATCTTTAAGG GGAGGAACCA GAGTGCTGAA				2132
GGAATGGAAC TCCATCTCCG TGTGTGCAGG GAGACTGGGT AGGAAAGAGG AAGCAAATAG				2192
AAGAGAGAGG TTGAAAAACA AAATGGGTGA CTTGATTGGT GATTAGGTGG TGGTAGAGAA				2252
GCAAGTAAAA AGGCTAAATG GAAGGGCAAG TTTCCATCAT CTATAGAAAG CTATGTAAGA				2312
CAAGGACTCC CCTTTTTTTC CCAAAGGCAT TGTA AAAAGA ATGAAGTCTC CTTAGAAAAA				2372
AAATTATACC TCAATGTCCC CAACAAGATT GCTTAATAAA TTGTGTTTCC TCCAAGCTAT				2432
TCAATICTTT TAACTGTTGT AGAAGAGAAA ATGTTACAAA TATATTTAGT TGTA AACCAA				2492
GTGATCAAAAC TACATATTGT AAAGCCCAT TTTAAAATAC ATTGTATATA TGTGTATGCA				2552
CAGTAAAAAT GGAAACTATA TTGACCTAAA AAAAAAAAAA GGAAACCACC CTTAGGCAGG				2612
CAGGACATGC TCTTCAGAAC TCTGCTCTTC AGAGTTCCAA AGAAGGGATA AAACATCTTT				2672
TAT				2675

## ( 2 ) INFORMATION FOR SEQ ID NO:5:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 188 amino acids
- ( B ) TYPE: amino acid
- ( D ) TOPOLOGY: linear

## ( ii ) MOLECULE TYPE: protein

## ( iii ) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Asp Cys Arg Lys Met Val Arg Phe Ser Tyr Ser Val Ile Trp Ile  
 1 5 10 15  
 Met Ala Ile Ser Lys Ala Phe Glu Leu Gly Leu Val Ala Gly Leu Gly

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20	25	30
His Gln Gln Phe Ala Arg Pro Ser Arg Gly Asp Leu Ala Phe Arg Asp 35 40 45		
Asp Ser Ile Trp Pro Gln Gln Gln Pro Ala Ile Arg Pro Arg Ser Ser 50 55 60		
Gln Arg Val Leu Pro Met Gly Ile Gln His Ser Lys Gln Leu Asn Arg 65 70 75 80		
Thr Cys Cys Leu Asn Gln Gly Thr Cys Met Leu Gly Ser Phe Cys Ala 85 90 95		
Cys Pro Pro Ser Phe Tyr Gly Arg Asn Cys Gln His Asp Val Arg Lys 100 105 110		
Gln Asn Cys Gly Ser Val Pro His Asp Thr Trp Leu Pro Lys Lys Cys 115 120 125		
Ser Leu Cys Lys Cys Trp His Gly Gln Leu Arg Cys Phe Pro Gln Ala 130 135 140		
Phe Leu Pro Gly Cys Asp Gly Leu Val Met Asp Gln His Leu Val Ala 145 150 155 160		
Ser Arg Thr Pro Gln Leu Pro Pro Ser Ala Arg Thr Thr Thr Phe Met 165 170 175		
Leu Ala Gly Ile Cys Leu Ser Ile Gln Ser Tyr Tyr 180 185		

What is claimed is:

1. A polypeptide free of other naturally associated proteins, wherein the polypeptide comprises the amino acid sequence set forth in SEQ ID NO:5. 30
2. A polypeptide bound to a solid support, wherein the polypeptide comprises the amino acid sequence set forth in SEQ ID NO:5.

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